The cells are then sonicated, thus releasing phage that have successfully entered the target cells (thus protecting them from the polyclonal anti-M13 antiserum). These phage can, if desired, be amplified, experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis), and the selective cycle will be iteratively applied for, e.g., 3 - times. Individual phage from the final cycle can then be characterized with respect to their relative invasiveness. The best candidates can then be combined with gene III fusions that encode pathogenic epitopes of interest. These phage can be injected into mice and tested for their relative abilities to induce a CTL response to the pathogenic antigens.

Bacteriophage vaccine vehicles evolved for activity in mice according to the above methods will establish the principles for the evolution of similar vehicles for potent human vaccines. The ability to induce more rapid and potent CTL and neutralizing antibody responses with such vehicles is an important new tool for the evolution of improved countermeasures against pathogens of interest.

Evolution Of Improved Immunomodulatory Sequences

Cytokines can dramatically influence macrophage activation and T_H1/ T_H2 cell differentiation, and thereby the outcome of infectious diseases. In addition, recent studies strongly suggest that DNA itself can act as adjuvant by activating the cells of the immune system. Specifically, unmethylated CpG-rich DNA sequences were shown to enhance T_H1 cell differentiation, activate cytokine synthesis by monocytes and induce proliferation of B lymphocytes. The invention thus provides methods for enhancing the immunomodulatory properties of genetic vaccines (a) by evolving the stimulatory properties of DNA itself and (b) by evolving genes encoding cytokines and related molecules that are involved in immune system regulation. These genes are then used in genetic vaccine vectors.

Of particular interest are IFN-(x and IL-12, which skew immune responses towards a T helper I (T_H1) cell phenotype and, thereby, improve the host's capacity to counteract pathogen invasions. Also provided are methods of obtaining improved immunomodulatory nucleic acids that are capable of inhibiting or enhancing activation, differentiation, or anergy of antigen-specific T cells. Because of the limited information about the structures and mechanisms that regulate these events, molecular breeding C71 techniques of the invention provide much faster solutions than rational design.

The methods of the invention typically involve the use of stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide

reassembly or other methods to create a library of experimentally generated (in vitro &/or in vivo) polynucleotides. The library is then screened to identify experimentally generated polynucleotides in the library, when included in a genetic vaccine vector or administered in conjunction with a genetic vaccine, are capable of enhancing or otherwise altering an immune response induced by the vector. The screening step, in some embodiments, can involve introducing a genetic vaccine vector that includes the experimentally generated polynucleotides into mammalian cells and determining whether the cells, or culture medium obtained by growing the cells, is capable of modulating an immune response.

Optimized recombinant vector modules obtained through polynucleotide reassembly (&/or one or more additional directed evolution methods described herein) are useful not only as components of genetic vaccine vectors, but also for production of polypeptides, e.g., modified cytokines and the like, that can be administered to a mammal to enhance or shift an immune response. Polynucleotide sequences obtained using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention can be used as a component of a genetic vaccine, or can be used for production of cytokines and other immunomodulatory polypeptides that are themselves used as therapeutic or prophylactic reagents. If desired, the sequence of the optimized immunomodulatory polypeptide-encoding polynucleotides can be determined and the deduced amino acid sequence used to produce polypeptides using methods known to those of skill in the art.

Immunostimulatory DNA Sequences

The invention provides methods of obtaining polynucleotides that are immunostimulatory when introduced into a mammal. Oligonucleotides that contain hexamers with a central CpG flanked by two 5' purines (GpA or ApA) and two 3' pyrimidines (TpC or TpT) efficiently induce cytokine synthesis and B cell proliferation (Krieg et al. (1995) Nature 374: 546; Klinman et al. (1996) Proc. Nat'l. Acad. Sci. USA 93: 2879; Pisetsky (1996) Immunity 5: 303-10) *in vitro* and act as adjuvants *in vivo*. Genetic vaccine vectors in which immunostimulatory sequence- (ISS) containing oligos are inserted have increased capacity to enhance antigen-specific antibody responses after DNA vaccination. The minimal length of an ISS oligonucleotide for functional activity *in vitro* is eight (Klinman et al., supra.). Twenty-mers with three CG motifs were found to be significantly more efficient in inducing cytokine synthesis than a 15- mer with two CG motifs (Id.). GGGG tetrads have been

suggested to be involved in binding of DNA to cell surfaces (macrophages express receptors. for example scavenger receptors, that bind DNA) (Pisetsky et al., supra.).

According to the invention, a library is generated by subjecting to reassembly (&/or one or more additional directed evolution methods described herein) random DNA (e.g., fragments of human, murine, or other genomic DNA), oligonucleotides that contain known ISS, poly A, C, G or T sequences, or combinations thereof. The DNA, which includes at least first and second forms which differ from each other in two or more nucleotides, are reassembled (&/or subjected to one or more directed evolution methods described herein) to produce a library of experimentally generated polynucleotides.

The library is then screened to identify those experimentally generated polynucleotides that exhibit immunostimulatory properties, For example, the library can be screened for induction cytokine production *in vitro* upon introduction of the library into an appropriate cell type. A diagram of this procedure is shown, described &/or referenced herein (including incorporated by reference). Among the cytokines that can be used as an indicator of immunostimulatory activity are, for example, IL-2, IL-4, IL-5, IL- 6, IL- 10, IL- 12, IL-13, IL- 15, and IFN-γ. One can also test for changes in ratios of IL-4/IFN- y, IL-4/IL-2, IL-5/ IFN-γ, IL-5/IL-2, IL- I 3/ IFN-γ, IL- 13/IL-2. An alternative screening method is the determination of the ability to induce proliferation of cells involved in immune responses, such as B cells, T cells, monocytes/macrophages, total PBL, and the like. Other screens include detecting induction of APC activation based on changes in expression levels of surface antigens, such as B7-1 (CD80), B7-2 (CD86), MHC class I and II, and CD14.

Other useful screens include identifying, experimentally generated polynucleotides that induce T cell proliferation. Because ISS sequences induce B cell activation, and because of several homologies between surface antigens expressed by T cells and B cells, polynucleotides can be obtained that have stimulatory activities on T cells.

Libraries of experimentally generated polynucleotides can also be screened for improved CTL and antibody responses *in vivo* and for improved protection from infection, cancer, allergy or autoimmunity. Experimentally generated polynucleotides that exhibit the desired property can be recovered from the cell and, if further improvement is desired, the reassembly (optionally in combination with other directed evolution methods described herein) and screening, can be repeated. Optimized ISS sequences can used as an adjuvant

separately from an actual vaccine, or the DNA sequence of interest can be fused to a genetic vaccine vector.

Cytokines, Chemokines, And Accessory Molecules

The invention also provides methods for obtaining optimized cytokines, cytokine antagonists, chemokines, and other accessory molecules that direct, inhibit, or enhance immune responses. For example, the methods of the invention can be used to obtain genetic vaccines and other reagents (e.g., optimized cytokines, and the like) that, when administered to a mammal, improve or alter an immune response. These optimized immunomodulators are useful for treating infectious diseases, as well as other conditions such as inflammatory disorders, in an antigen non-specific manner.

For example, the methods of the invention can be used to develop optimized immunomodulatory molecules for treating allergies. The optimized immunomodulatory molecules can be used alone or in conjunction with antigen-specific genetic vaccines to prevent or treat allergy. Four basic mechanisms are available by which one can achieve specific immunotherapy of allergy. First, one can administer a reagent that causes a decrease in allergen-specific T_H2 cells. Second, a reagent can be administered that causes an increase in allergen-specific T_H1 cells. Third, one can direct an increase in suppressive $CD8^+$ T cells.

Finally, allergy can be treated by inducing anergy of allergen-specific T cells. In this Example, cytokines are optimized using the methods of the invention to obtain reagents that are effective in achieving one or more of these immunotherapeutic goals. The methods of the invention are used to obtain anti-allergic cytokines that have one or more properties such as improved specific activity, improved secretion after introduction into target cells, are effective at a lower dose than natural cytokines, and fewer side effects. Targets of particular interest include interferon-α/γ, IL-10, IL-12, and antagonists of IL-4 and IL-13.

The optimized immunomodulators, or optimized experimentally generated polynucleotides that encode the immunomodulators, can be administered alone, or in combination with other accessory molecules. Inclusion of optimal concentrations of the appropriate molecules can enhance a desired immune response, and/or direct the induction or repression of a particular type of immune response. The polynucleotides that encode the optimized molecules can be included in a genetic vaccine vector, or the optimized molecules encoded by the genes can be administered as polypeptides.

In the methods of the invention, a library of experimentally generated polynucleotides that encode immunomodulators is created by subjecting substrate nucleic acids to a reassembly (&/or one or more additional directed evolution methods described herein) protocol, such as stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly or other method known to those of skill in the art. The substrate nucleic acids are typically two or more forms of a nucleic acid that encodes an immunomodulator of interest.

Cytokines are among the immunomodulators that can be improved using the 0 methods of the invention. Cytokine synthesis profiles play a crucial role in the capacity of the host to counteract viral, bacterial and parasitic infections, and cytokines can dramatically influence the efficacy of genetic vaccines and the outcome of infectious diseases. Several cytokines, for example IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, IL-15, IL-16, IL-17, IL-18, G-CSF, GM-CSF, IFN-α, IFN- γ, TGF-β, TNF-α, TNF-β, IL-20 (MDA-7), and flt-3 ligand have been shown stimulate immune responses *in vitro* or *in vivo*. Immune functions that can be enhanced using appropriate cytokines include, for example, B cell proliferation, Ig synthesis, Ig isotype switching, T cell proliferation and cytokine synthesis, differentiation of T_H1 and T_H2 cells, activation and proliferation of CTLs, activation and cytokine production by monocytes/macrophages/ dendritic cells, and differentiation of dendritic cells from monocytes/macrophages.

In some embodiments, the invention provides methods of obtaining optimized immummomodulators that can direct an immune response towards a T_H1 or a T_H2 response. The ability to influence the direction of immune responses in this manner is of great importance in development of genetic vaccines. Altering the type of T_H response can fundamentally change the outcome of an infectious disease. A high frequency of T_H1 cells generally protects from lethal infections with intracellular pathogens, whereas a dominant T_H2 phenotype often results in disseminated, chronic infections. For example, in human, the T_H1 phenotype is present in the tuberculoid (resistant) form of leprosy, while the T_H2 phenotype is found in lepromatous, multibacillary (susceptible) lesions (Yamamura et al. (1991) Science 254: 277). Late-stage AIDS patients have the T_H2 phenotype. Studies in family members indicate that survival from meningococcal septicemia depends on the cytokine synthesis profile of PBL, with high IL-10 synthesis being associated with a high risk of lethal outcome and high TNF-α being associated with a low risk. Similar examples are

found in mice. For example, BALB/c mice are susceptible to Leishmania major infection; these mice develop a disseminated fatal disease with a T_H2 phenotype. Treatment with anti-IL-4 monoclonal antibodies or with IL-12 induces a T_H1 response, resulting in healing. Anti-interferon- γ monoclonal antibodies exacerbate the disease. For some applications, the immune response can be directed in the direction of a T_H2 response.

For example, where increased mucosal immunity is desired, including protective immunity, enhancing the T_H2 response can lead to increased antibody production, particularly IgA. T helper (T_H) cells are probably the most important regulators of the immune system. T_H cells are divided into two subsets, based on their cytokine synthesis pattern (Mosmann and Coffman (1989) Adv. Immunol. 46: 111). T_H1 cells produce high levels of the cytokines IL-2 and IFN-γ and no or minimal levels of IL-4, IL-5 and IL- 13. In contrast, T_H2 cells produce high levels of IL-4, IL-5 and IL-13, and IEL-2 and IFN-γ production is minimal or absent. T_H1 cells activate macrophages, dendritic cells and augment the cytolytic activity of $CD8^+$ cytotoxic T lymphocytes and natural killer (NK) cells (Paul (1994) Cell 76: 241), whereas T_H2 cells provide efficient help for B cells and also mediate allergic responses due to the capacity of T_H2 cells to induce IgE isotype switching and differentiation of B cells into IgE secreting cells (Punnonen et al. (1993) Proc. Nat'l. Acad. Sci. USA 90: 3730).

The screening methods for improved cytokines, chemokines, and other accessory molecules are generally based on identification of modified molecules that exhibit improved specific activity on target cells that are sensitive to the respective cytokine, chemokine, or other accessory molecules. A library of recombinant cytokine, chemokine, or accessory molecule nucleic acids can be expressed on phage or as purified protein and tested using *in vitro* cell culture assays, for example. Importantly, when analyzing the recombinant nucleic acids as components of DNA vaccines, one can identify the most optimal DNA sequences (in addition to the functions of the protein products) in terms of their immunostimulatory properties, transfection efficiency, and their capacity to improve the stabilities of the vectors. The identified optimized recombinant nucleic acids can then be subjected to new rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection.

In one embodiment of the invention, cytokines are evolved that direct differentiation of T_H1 cells. Because of their capacities to skew immune responses towards a T_H1

phenotype, the genes encoding interferon-α (IFN-α) and interleukin-12 (IL-12) can be substrates for reassembly (&/or one or more additional directed evolution methods described herein) and selection in order to obtain maximal specific activity and capacity to act as adjuvants in genetic vaccinations. IFN- α is an exemplary target for optimization using the methods of the invention because of its effects on the immune system, tumor cells growth and viral replication. Due to these activities, IFN- α was the first cytokine to be used in clinical practice. Today, IFN- α is used for a wide variety of applications, including several types of cancers and viral diseases. IFN- α also efficiently directs differentiation of human T cells into T_H1 phenotype (Parronchi et al. (1992) J Immunol. 149: 2977). However, it has not been thoroughly investigated in vaccination models, because, in contrast to human systems, it does not affect T_H1 differentiation in mice.

The species difference was recently explained by data indicating that, like IL-12, IFN- α induces STAT4 activation in human cells but not in murine cells, and STAT4 has been shown to be required in IL-12 mediated T_H1 differentiation (Thierfelder et al. (1996) Nature 382: 171).

Family stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is an exemplary method for optimizing IFN- α, using as substrates the mammalian IFN- α. genes, which are 85% - 97% homologous. Greater 10^{26} distinct recombinants can be generated from the natural diversity in these genes. To allow rapid parallel analysis of recombinant interferons, one can employ high throughput methods for their expression and biological assay as fusion proteins on bacteriophage.

Recombinants with improved potency and selectivity profiles are being selectively bred for improved activity. Variants which demonstrate improved binding to IFN- α receptors can be selected for further analysis using a screen for mutants with optimal capacity to direct T_H1 differentiation. More specifically, the capacities of IFN- α mutants to induce IL-2 and IFN-γ production in *in vitro* human T lymphocyte cultures can be studied by cytokine-specific ELISA and cytoplasmic cytokine staining and flow cytometry.

IL-12 is perhaps the most potent cytokine that directs T_H1 responses, and it has also been shown to act as an adjuvant and enhance T_H1 responses following genetic vaccinations (Kim et al. (1997) J Immunol. 15 8: 816). IL-12 is both structurally and functionally a unique cytokine. It is the only heterodimeric cytokine known to date, composed of a 35 kD light

chain (p35) and a 40 kD heavy chain (p40) (Kobayashi et al (1989) J Exp. Med. 170: 827; Stem et al. (1990) Proc. Nat'l. Acad. Sci. USA 87: 6808).

Recently Lieschke et al. ((1997) Nature Biotech. 15: 3 5) demonstrated that a fusion between p35 and p40 genes results in a single gene that has activity comparable to that of the two genes expressed separately. These data indicate that it is possible to reassemble IL-12 genes as one entity, which is beneficial in designing the reassembly protocol (optionally in combination with other directed evolution methods described herein). Because of its T cell growth promoting activities, one can use normal human peripheral blood T cells in the selection of the most active IL-12 genes, enabling direct selection of IL-12 mutants with the most potent activities on human T cells. IL-12 mutants can be expressed in CHO cells, for example, and the ability of the supernatants to induce T cell proliferation determined. The concentrations of IL-12 in the supernatants can be normalized based on a specific ELISA that detects a tag fused to the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) IL- 12 molecules.

Incorporation of evolved IFN- α and/or IL-12 genes into genetic vaccine vectors is expected to be safe. The safety of IFN- α has been demonstrated in numerous clinical studies and in everyday hospital practice. A Phase II trial of IL-12 in the treatment of patients with renal cell cancer resulted in several unexpected adverse effects (Tahara et al. (1995) Human Gene Therapy 6: 1607). However, IL-12 gene as a component of genetic vaccines alms at high local expression levels, whereas the levels observed in circulation are minimal compared to those observed after systemic bolus injections. In addition, some of the adverse effects of systemic IL-12 treatments are likely to be related to its unusually long half-life (up to 48 hours in monkeys). stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly may allow selection for a shorter half- life, thereby reducing the toxicity even after high bolus doses.

In other cases, genetic vaccines that can induce T_H2 responses can be used, especially when improved antibody production is desired. As an example, IL-4 has been shown to direct differentiation of T_H2 cells (which produce high levels of IL-4, IL-5 and IL-13, and mediate allergic immune responses). Immune responses that are skewed towards T_H2 phenotype may be preferred when genetic vaccines are used to immunize against autoimmune diseases prophylactically. T_H1 responses may be desired when the vaccines are used to treat and modulate existing autoimmune responses, because autoreactive T cells are generally of T_H1

phenotype (Liblau et al. (1995) Immunol. Today 16:34- 38). IL-4 is also the most potent cytokine in induction of IgE synthesis; IL-4 deficient mice are unable to produce IgE. Asthma and allergies are associated with an increased frequency of IL-4 producing cells, and are genetically linked to the locus encoding IL-4, which is on chromosome 5 (in close proximity to genes encoding IL-3, IL-5, IL-9, IL-13 and GM-CSF). IL-4, which is produced by activated T cells, basophils and mast cells, is a protein that has 153 amino acids and two potential N-glycosylation sites. Human IL-4 is only approximately 50% identical to mouse IL-4, and IL-4 activity is species-specific. In human, IL- 13 has activities similar to those of IL-4, but IL-13 is less potent than IL-4 in inducing IgE synthesis. IL-4 is the only cytokine known to direct T_H2 differentiation.

Improved IL-2 agonists are also useful in directing T_H2 cell differentiation, whereas improved IL-4 antagonists can direct T_H1 cell differentiation. Improved IL-4 agonists and antagonists can be generated by the reassembly (optionally in combination with other directed evolution methods described herein) of IL-4 or soluble IL-4 receptor. The IL-4 receptor consists of an IL-4R α-chain (140 kD high-affinity binding unit) and an IL-2R γ-chain (these cytokine receptors share a common 7-chain). The IL-4R α-chain is shared by IL-4 and IL- 13 receptor complexes. Both IL-4 and IL-13 induce phosphorylation of the IL- 4R α-chain, but expression of IL-4R α-chain alone on transfectants is not sufficient to provide a functional IL-4R. Soluble IL-4 receptor currently in clinical trials for the treatment of allergies. Using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention, one can evolve a soluble IL- 4 receptor that has improved affinity for IL-4. Such receptors are useful for the treatment of asthma and other T_H2 cell mediated diseases, such as severe allergies. The reassembly (optionally in combination with other directed evolution methods described herein) reactions can take advantage of natural diversity present in cDNA libraries from activated T cells from human and other primates. In a typical embodiment, a experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) IL-4R α-chain library is expressed on a phage, and mutants that bind to IL-4 with improved affinity are identified. The biological activity of the selected mutants is then assayed using cell-based assays.

IL-2 and IL-15 are also of particular interest for use in genetic vaccines. IL-2 acts as a growth factor for activated B and T cells, and it also modulates the functions of NK-cells. IL-

2 is predominantly produced by T_H1-like T cell clones, and, therefore, it is considered mainly to function in delayed type hypersensitivity reactions. However, IL-2 also has potent, direct effects on proliferation and Ig-synthesis by B cells. The complex immunoregulatory properties of IL-2 are reflected in the phenotype of IL- 2 deficient mice, which have high mortality at young age and multiple defects in their immune functions including spontaneous development of inflammatory bowel disease. IL- 15 is a more recently identified cytokine produced by multiple cell types. IL- 15 shares several, but not all, activities with IL-2. Both IL-2 and IL-15 induce B cell growth and differentiation. However, assuming that IL-15 production in IL-2 deficient mice is normal, it is clear that IL-15 cannot substitute for the function of IL-2 *in vivo*, since these mice have multiple immunodeficiencies. IL-2 has been shown to synergistically enhance IL-10- induced human Ig production in the presence of anti-CD40 mAbs, but it antagonized the effects of IL-4. IL- 2 also enhances IL-4-dependent IgE synthesis by purified B cells. On the other hand, IL-2 was shown to inhibit IL-4-dependent murine IgG1 and IgE synthesis both *in vitro* and *in vivo*. Similarly, IL-2 inhibited IL-4-dependent human IgE synthesis by unfractionated human PBMC, but the effects were less significant than those of IFN- α or IFN-γ. Due to their capacities to activate both B and T cells, IL-2 and IL- 15 are useful in vaccinations. In fact, IL-2, as protein and as a component of genetic vaccines, has been shown to improve the efficacy of the vaccinations. Improving the specific activity and/or expression levels/kinetics of IL-2 and IL- 15 through use of the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention increases the advantageous effects compared to wild-type IL-2 and IL-15.

Another cytokine of particular interest for optimization and use in genetic vaccines according to the methods of the invention is interleukin-6. IL-6 is a monocyte- derived cytokine that was originally described as a B cell differentiation factor or B cell stimulatory factor-2 because of its ability to enhance Ig levels secreted by activated B cells.

IL-6 has also been shown to enhance IL-4-induced I-E synthesis. It has also been suggested that IL-6 is an obligatory factor for human IgE synthesis, because neutralizing anti-IL-6 mAbs completely blocked IL-4-induced IgE synthesis. IL-6 deficient mice have impaired capacity to produce IgA. Because of its potent activities on the differentiation of B cells, IL- 6 can enhance the levels of specific antibodies produced following vaccination. It is particularly useful as a component of DNA vaccines because high local concentrations can be

achieved, thereby providing the most potent effects on the cells adjacent to the transfected cells expressing the immunogenic antigen. IL-6 with improved specific activity and/or with improved expression levels, obtained by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly, will have more beneficial effects than the wild-type IL-6.

Interleukin-8 is another example of a cytokine that, when modified according to the methods of the invention, is useful in genetic vaccines. IL-8 was originally identified as a monocyte-derived neutrophil chemotactic and activating factor. Subsequently, IL-8 was also shown to be chemotactic for T cells and to activate basophils resulting in enhanced histamine and leukotriene release from these cells. Furthermore, IL-8 inhibits adhesion of neutrophils to cytokine-activated endothelial cell monolayers, and it protects these cells from neutrophil-mediated damage. Therefore, endothelial cell derived IL-8 was suggested to 3 _31 attenuate inflammatory events occurring in the proximity of blood vessel walls. IL-8 also modulates immunoglobulin production, and inhibits IL-4-induced IgG4 and IgE synthesis by both unfractionated human PBMC and purified B cells *in vitro*. This inhibitory effect was independent of IFN- α, IFN-γ or prostaglandin E2. In addition, IL-8 inhibited spontaneous IgE synthesis by PBMC derived from atopic patients. Due to its capacity to attract inflammatory cells, IL-8, like other chemotactic agents, is useful in potentiating the functional properties of vaccines, including DNA vaccines (acting as an adjuvant). The beneficial effects of IL-8 can be improved by using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention to obtain IL-8 with improved specific activity and/or with improved expression in target cells.

Interleukin-5, and antagonists thereof, can also be optimized using the methods of the invention for use in genetic vaccines. IL-5 is primarily produced by T_H2-type T cells and appears to play an important role in the pathogenesis of allergic disorders because of its ability to induce eosinophilia. IL-5 acts as an eosinophil differentiation and survival factor in both mouse and man. Blocking IL-5 activity by use of neutralizing monoclonal antibodies strongly inhibits pulmonary eosinophilia and hyperactivity in mouse models, and IL-5 deficient mice do not develop eosinophilia. These data also suggest that IL-5 antagonists may have therapeutic potential in the treatment of allergic eosinophilia.

IL-5 has also been shown to enhance both proliferation of, and Ig synthesis by, activated mouse and human B cells. However, other studies suggested that IL-5 has no effect on proliferation of human B cells, whereas it activated eosinophils. IL-5 apparently is not crucial for maturation or differentiation of conventional B cells, because antibody responses in IL-5 deficient mice are normal. However, these mice have a developmental defect in their $CD5^+$ B cells indicating that IL-5 is required for normal differentiation of this B cell subset in mice. At suboptimal concentrations of IL-4, IL-5 was shown to enhance IgE synthesis by human B cells *in vitro*. Furthermore, a recent study suggested that the effects of IL-5 on human B cells depend on the mode of B cell stimulation. IL-5 significantly enhanced IgM synthesis by B cells stimulated with *Moraxella catarrhalis*. In addition, IL-5 synergized with suboptimal concentrations of IL-2, but had no effect on I- synthesis by SAC-activated B cells. Activated human B cells also expressed IL-5 mRNA suggesting that IL-5 may also regulate B cell function, including I-E synthesis, by autocrine mechanisms.

The invention provides methods of evolving an IL-5 antagonist that efficiently binds to and neutralizes IL-5 or its receptor. These antagonists are useful as a component of vaccines used for prophylaxis and treatment of allergies. Nucleic acids encoding IL-5, for example, from human and other mammalian species, are experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) and screened for binding to immobilized IL-5R for the initial screening. Polypeptides that exhibit the desired effect in the initial screening assays can then be screened for the highest biological activity using assays such as inhibition of growth of IL-5 dependent cells lines cultured in the presence of recombinant wild-type IL-5. Alternatively, experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) IL-5R α - chains are screened for improved binding to IL-5.

Tumor necrosis factors (α and β) and their receptors are also suitable targets for modification and use in genetic vaccines. TNF-α, which was originally described as cachectin because of its ability to cause necrosis of tumors, is a 17 kDa protein that is produced in low quantities by almost all cells in the human body following activation. TNF-α acts as an endogenous pyrogen and induces the synthesis of several proinflammatory cytokines, stimulates the production of acute phase proteins, and induces proliferation of fibroblasts. TNF-α plays a major role in the pathogenesis of endotoxin shock. A membrane-bound form of TNF-α (mTNF- α), which is involved in interactions between B- and T-cells,

is rapidly upregulated within four hours of T cell activation. mTNF-α plays a role in the polyclonal B cell activation observed in patients infected with HIV. Monoclonal antibodies specific for mTNF-α. or the p55 TNF-α receptor strongly inhibit IgE synthesis induced by activated $CD4^+$ T cell clones or their membranes. Mice deficient for p55 TNF-αR are resistant to endotoxic shock, and soluble TNF-αR prevents autoimmune diabetes mellitus in NOD mice. Phase III trials using sTNF-αR in the treatment of rheumatoid arthritis are in progress, after promising results obtained in the phase II trials.

The methods of the invention can be used to, for example, evolve a soluble TNF-αR that has improved affinity, and thus is capable of acting as an antagonist for TNF activity. Nucleic acids that encode TNF-αR and exhibit sequence diversity, such as the natural diversity observed in cDNA libraries from activated T cells of human and other primates, are experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis). The experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) nucleic acids are expressed, e.g., on phage, after which mutants are selected that bind to TNF-α with improved affinity. If desired, the improved mutants can be subjected to further assays using biological activity, and the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes can be subjected to one or more rounds of reassembly (optionally in combination with other directed evolution methods described herein) and screening.

Another target of interest for application of the methods of the invention is interferon-γ, and the evolution of antagonists of this cytokine. The receptor for IFN-γ consists of a binding component glycoprotein of 90 kD, a 228 amino acid extracellular portion, a transmembrane region, and a 222 amino acid intracellular region. Glycosylation is not required for functional activity. A single chain provides high affinity binding (10^{-9}-10^{-10} M), but is not sufficient for signaling. Receptor components dimerize upon ligand binding.

The mouse IFN-γ receptor is 53% identical to that of mouse at the amino acid level. The human and mouse receptors only bind human and mouse IFN-γ, respectively. Vaccinia, cowpox and camelpox viruses have homologues of sIFN- γ R, which have relatively low amino acid sequence similarity (~20%), but are capable of efficient neutralization of IFN-γ *in vitro*. These homologues bind human, bovine, rat (but not mouse) IFN-γ, and may have *in vivo* activity as IFN- γ antagonists. All eight cysteines are conserved in human, mouse,

myxoma and Shope fibroma virus (6 in vaccinia virus) IFN- γ R polypeptides, indicating similar 3-D structures. An extracellular portion of m IFN- γR with a kD of 100-300 pM has been expressed in insect cells. Treatment of NZB/W mice (a mouse model of human SLE) with msIFN- γ receptor (100 mg/three times a week i.p.) inhibits the onset of glomerulonephritis. All mice treated with sIFN- γ or anti- IFN- γ niAbs were alive 4 weeks after the treatment was discontinued, compared with 50% in a placebo group, and 78% of IFN- γ -treated mice died.

The methods of the invention can be used to evolve soluble IFN-γR receptor polypeptides with improved affinity, and to evolve IFN-γ with improved specific activity and improved capacity to activate cellular immune responses. In each case nucleic acids encoding the respective polypeptide, and which exhibit sequence diversity (e.g., that observed in cDNA libraries from activated T cells from human and other primates), are subjected to reassembly (&/or one or more additional directed evolution methods described herein) and screened to identify those recombinant nucleic acids that encode a polypeptide having improved activity. In the case of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) IFN-γR, the library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) nucleic acids can be expressed on phage, which are screened to identify mutants that bind to IFN-γ with improved affinity. In the case of IFN-γ, the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) library is analyzed for improved specific activity and improved activation of the immune system, for example, by using activation of monocytes/macrophages as an assay. The evolved IFN-γ molecules can improve the efficacy of vaccinations (e.g. when used as adjuvants). Diseases that can be treated using high-affinity sIFN-γR polypeptides obtained using the methods of the invention include, for example, multiple sclerosis, systemic lupus erythematosus (SLE), organ rejection after treatment, and graft versus host disease. Multiple sclerosis, for example, is characterized by increased expression of IFN-γ in the brain of the patients, and increased production of IFN-γ by patients' T cells *in vitro*. IFN-γ treatment has been shown to significantly exacerbate the disease (in contrast to EAE in mice).

Transforming growth factor (TGF)-β is another cytokine that can be optimized for use in genetic vaccines using the methods of the invention. TGF- β has growth regulatory activities on essentially all cell types, and it has also been shown to have complex

modulatory effects on the cells of the immune system. TGF- β inhibits proliferation of both B and T cells, and it also suppresses development of and differentiation of cytotoxic T cells and NK cells, TGF- β has been shown to direct IgA switching in both murine and human B cells. It was also shown to induce germline a transcription in murine and human B cells, supporting the conclusion that TGF- β can specifically induce IgA switching.

Due to its capacity to direct IgA switching, TGF- β is useful as a component of DNA vaccines which aim at inducing potent mucosal immunity, *e.g.* vaccines for diarrhea. Also, because of its potent anti-proliferative effects TGF- β is useful as a component of therapeutical cancer vaccines. TGF- β with improved specific activity and/or with improved expression levels/kinetics will have increased beneficial effects compared to the wild-type TGF- β.

Cytokines that can be optimized using the methods of the invention also include granulocyte colony stimulating factor (G-CSF) and granulocyte/macrophage colony stimulating factor (GM-CSF). These cytokines induce differentiation of bone marrow stem cell into granulocytes/macrophages. Administration of G-CSF and GM-CSF significantly improve recovery from bone marrow (BM) transplantation and radiotherapy, reducing infections and time the patients have to spend in hospitals. GM-CSF enhances antibody production following DNA vaccination. G-CSF is a 175 amino acid protein, while GM-CSF has 127 amino acids. Human G-CSF is 73% identical at the amino acid level to murine G-CSF and the two proteins show species cross-reactivity. G-CSF has a homodimeric receptor (dimeric with kD of ~200 pM, monomeric ~2.4 nM), and the receptor for GM-CSF is a three subunit complex. Cell lines transfected with cDNA encoding G-CSF R proliferate in response to G-CSF. Cell lines dependent of GM-CSF available (such as TF-1). G-CSF is nontoxic and is presently working very well as a drug. However, the treatment is expensive, and more potent G-CSF might reduce the cost for patients and to the health care. Treatments with these cytokines are typically short-lasting and the patients are likely to never need the same treatment again reducing likelihood of problems with immunogenicity.

The methods of the invention are useful for evolving G-CSF and/or GM-CSF which have improved specific activity, as well as other polypeptides that have G-CSF and/or GM-CSF activity. G-CSF and/or GM-CSF nucleic acids having sequence diversity, e.g., those obtained from cDNA libraries from diverse species, are experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) to create a

library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) G-CSF and/or GM-CSF genes. These libraries can be screened by, for example, picking colonies, transfecting the plasmids into a suitable host cell (*e.g.*, CHO cells), and assaying the supernatants using receptor-positive cell lines. Alternatively, phage display or related techniques can be used, again using receptor-positive cell lines. Yet another screening method involves transfecting the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes into G-CSF/GM- CSF-dependent cell lines. The cells are grown one cell per well and/or at very low density in large flasks, and the cells that grow fastest are selected. Experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes from these cells are isolated; if desired, these genes can be used for additional rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection.

Ciliary neurotrophic factor (CNTF) is another suitable target for application of the methods of the invention. CNTF has 200 amino acids which exhibit 80% sequence identity between rat and rabbit CNTF polypeptides. CNTF has IL-6-like inflammatory effects, and induces synthesis of acute phase proteins. CNTF is a cytosolic protein which belongs to the IL-6/IL-11I/LIF/oncostatin M -family, and becomes biologically active only after becoming available either by cellular lesion or by an unknown release mechanism. CNTF is expressed by myelinating Schwann cells, astrocytes and sciatic nerves.

Structurally, CNTF is a dimeric protein, with a novel anti-parallel arrangement of the subunits. Each subunit adopts a double crossover four-helix bundle fold, in which two helices contribute to the dimer interface. Lys-155 mutants lose activity, and some Glu-153 mutants have 5-10 higher biological activity. The receptor for CNTF consists of a specific CNTF receptor chain, gp130, and a LIF- β receptor. The CNTFR α-chain lacks a transmembrane domain portion, instead being GPI-anchored. At high concentration, CNTF can mediate CNTFR-independent responses. Soluble CNTFR binds CNTF and thereafter can bind to LIFR and induce signaling through gp 130. CNTF enhances survival of several types of neurons, and protects neurons in an animal model of Huntington disease (in contrast to NGF, neurotrophic factor, and neurotrophin-3). CNTF receptor knockout mice have severe motor neuron deficits at birth, and CNTF knockout mice exhibit such deficits postnatally. CNTF also reduces obesity in mouse models. Decreased expression of CNTF is sometimes observed

in psychiatric patients. Phase I studies in patients with ALS (annual incidence ~1/100 000, 5% familiar cases, 90% die within 6 years) found significant side effects after doses higher than 5 mg/kg/day subcutaneously (including anorexia, weight loss, reactivation of herpes simplex virus (HSV1), cough, increased oral secretions). Antibodies against CNTF were detected in almost all patients, thus illustrating the need for alternative CNTF with different immunological properties.

The reassembly (&/or one or more additional directed evolution methods described herein) and screening methods of the invention can be used to obtain modified CNTF polypeptides that exhibit decreased immunogenicity *in vivo*; higher also obtainable using the methods. reassembly (optionally in combination with other directed evolution methods described herein) is conducted using nucleic acids encoding CNTF. In one aspect, an IL-6/LIF/(CNTF) hybrid is obtained by reassembly (optionally in combination with other directed evolution methods described herein) using an excess of oliconucleotides that encode to the receptor binding sites of CNTF. Phage display can then be used to test for lack of binding to the IL-6/LIF receptor.

This initial screen is followed by a test for high affinity binding to the CNTF receptor, and, if desired, functional assays using CNTF responsive cell lines. The experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) CNTF polypeptides can be tested to identify those that exhibit reduced immunogenicity upon administration to a mammal.

Another way in which the reassembly (&/or one or more additional directed evolution methods described herein) and screening methods of the invention can be used to optimize CNTF is to improve secretion of the polypeptide. When a CNTF cDNA is operably linked to a leader sequence of hNGF, only 35-40 percent of the total CNTF produced is secreted.

Target diseases for treatment with optimized CNTF, using either the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) gene in an expression vector as in DNA vaccines, or a purified protein, include obesity, amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease), diabetic neuropathy, stroke, and brain surgery.

Polynucleotides that encode chemokines can also be optimized using the methods of the invention and included in a genetic vaccine vector. At least three classes of chemokines are known, based on structure: C chemokines (such as lymphotactin), C-C chemokines (such

as MCP-1, MCP-2, MCP-3, MCP-4, MIP-1a, MIP-1b, RANTES), C-X-C chemokines (such as IL-8, SDF-1, ELR, Mig, IP 10) (Premack and Schall (1996) Nature Med. 2: 1174). Chemokines can attract other cells that mediate immune and inflammatory functions, thereby potentiating the immune response. Cells that are attracted by different types of chemokines include, for example, lymphocytes, monocytes and neutrophils. Generally, C-X-C chemokines are chemoattractants for neutrophils but not for monocytes, C-C chemokines attract monocytes and lymphocytes but not neutrophils, C chemokine attracts lymphocytes.

Genetic vaccine vectors can also include optimized experimentally generated polynucleotides that encode surface-bound accessory molecules, such as those that are involved in modulation and potentiation of immune responses. These molecules, which include, for example, B7-1 (CD80), B7-2 (CD86), CD40, ligand for CD40, CTLA-4, CD28, and CD 150 (SLAM), can be subjected to stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly to obtain variants have altered and/or improved activities.

Optimized experimentally generated polynucleotides that encode CD1 molecules are also useful in a genetic vaccine vector for certain applications. CD1 are nonpolymorphic molecules that are structurally and functionally related to MEC molecules. Importantly, CD I has MHC-like activities, and it can function as an antigen presenting molecule (Porcelli (1995) Adv. Immunol. 59: 1). CD1 is highly expressed on dendritic cells, which are very efficient antigen presenting cells. Simultaneous transfection of target cells with DNA vaccine vectors encoding CD1 and an antigen of interest is likely to boost the immune response. Because CD1 cells, in contrast to MHC molecules, exhibit limited allelic diversity in an outbred population (Porcelli, supra.), large populations of individuals with different genetic backgrounds can be vaccinated with one CD1 allele. The functional properties of CD1 molecules can be improved by the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methods of the invention.

Optimized recombinant TAP genes and/or gene products can also be included in a genetic vaccine vector. TAP genes and their optimization for various purposes are discussed in more detail below. Moreover, heat shock proteins (HSP), such as HSP70, can also be evolved for improved presentation and processing of antigens. HSP70 has been shown to act as adjuvant for induction of $CD8^+$ T cell activation and it enhances immunogenicity of specific antigenic peptides (Blachere et al. (1997) J Exp. Med. 186:1315-22). When HSP70

is encoded by a genetic vaccine vector, it is likely to enhance presentation and processing of antigenic peptides and thereby improve the efficacy of the genetic vaccines. stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly can be used to further improve the properties, including adjuvant activity, of heat shock proteins, such as HSP70.

Recombinantly produced cytokine, chemokine, and accessory molecule polypeptides, as well as antagonists of these molecules, can be used to influence the type of immune response to a given stimulus. However, the administration of polypeptides sometimes has shortcomings, including short half life, high expense, difficult to store (must be stored at 4°C), and a requirement for large volumes. Also, bolus injections can sometimes cause side effects. Administration of polynucleotides that encode the recombinant cytokines or other molecules overcomes most or all of these problems. DNA, for example, can be prepared in high purity, is stable, temperature resistant, noninfectious, easy to manufacture. In addition, polynucleotide-mediated administration of cytokines can provide long-lasting, consistent expression, and administration of polynucleotides in general is regarded as being safe.

The functions of cytokines, chemokines and accessory molecules are redundant and pleiotropic, and therefore can be difficult to determine which cytokines or cytokine combinations are the most potent in inducing and enhancing antigen specific immune responses following vaccination. Furthermore, the most useful combination of cytokines and accessory molecules is typically different depending on the type of immune C, response that is desired following vaccination. As an example, IL-4 has been shown to direct differentiation of T_H2 cells (which produce high levels of IL-4, IL-5 and IL-13, and mediate allergic immune responses), whereas IFN-γ and IL-12 direct differentiation of T_H1 cells (which produce high levels of IL-2 and IFN-γ), and mediate delayed type immune responses. Moreover, the most useful combination of cytokines and accessory molecules is also likely to depend on the antigen used in the vaccination. The invention provides a solution to this problem of obtaining an optimized genetic vaccine cocktail. Different combinations of cytokines, chemokines and accessory molecules are assembled into vectors using the methods described herein. These vectors are then screened for their capacity to induce immune responses *in vivo* and *in vitro*.

Large libraries of vectors, generated by polynucleotide (e.g. gene, promoter, enhancer, intron, & the like) reassembly (optionally in combination with other directed evolution

methods described herein) and combinatorial molecular biology, are screened for maximal capacity to direct immune responses towards, for example, a T_H1 or T_H2 phenotype, as desired. A library of different vectors can be generated by assembling different evolved promoters, (evolved) cytokines, (evolved) cytokine antagonists, (evolved) chemokines, (evolved) accessory molecules and immunostimulatory sequences, each of which can be prepared using methods described herein. DNA sequences and compounds that facilitate the transfection and expression can be included. If the pathogen(s) is known, specific DNA sequences encoding immunogenic antigens from the pathogen can be incorporated into these vectors providing protective immunity against the pathogen(s) (as in genetic vaccines).

Initial screening can be carried out *in vitro*. For example, the library can be introduced into cells which are tested for ability to induce differentiation of T cells capable of producing cytokines that are indicative of the type of immune response desired. For a T_H1 response, for example, the library is screened to identify experimentally generated polynucleotides that are capable of inducing T cells to produce IL-2 and IFN-γ, while screening for induction of T cell production of IL-4, IL-5, and IL-13 is performed to identify experimentally generated polynucleotides that favor a T_H2 response.

Screening can also be conducted *in vivo*, using animal models. For example, vectors produced using the methods of the invention can be tested for ability to protect against a lethal infection. Another screening method involves injection of Leishmania major parasites into footpads of BALB/c mice (nonhealer). Pools of plasmids are injected i.v., i.p. or into footpads of these mice and the size of the footpad swelling is followed. Yet another *in vivo* screening method involves detection of IgE levels after infection with Nippostrongylus brasiliensis. High levels indicate a T_H2 response, while low levels of IgE indicate a T_H1 response.

Successful results in animal models are easy to verify in humans. In vitro screening can be conducted to test for human T_H1 or T_H2 phenotype, or for other desired immune response. Vectors can also be tested for ability to induce protection against infection in humans. Because the principles of immune functions are similar in a wide variety of infections, immunostimulating DNA vaccine vectors may not only be useful in the treatment of a number of infectious diseases but also in prevention of the infections, when the vectors are delivered to the sites of the entry of the pathogen (e.g., the lung or gut).

Agonists Or Antagonists Of Cellular Receptors

The invention also provides methods for obtaining optimized experimentally generated polynucleotides that encode a peptide or polypeptide that can interact with a cellular receptor that is involved in mediating an immune response. The optimized experimentally generated polynucleotides can act as an agonist or an antagonist of the receptor.

Cytokine antagonists can be used as components of genetic vaccine cocktails

Blocking immunosuppressive cytokines, rather than adding single proinflammatory cytokines, is likely to potentiate the immune response in a more general manner, because several pathways are potentiated at the same time. By appropriate choice of antagonist, one can tailor the immune response induced by a genetic vaccine in order to obtain the response that is most effective in achieving the desired effect. Antagonists against any cytokine can be used as appropriate; particular cytokines of interest for blocking include, for example, IL-4, IL-13, IL-10, and the like.

The invention provides methods of obtaining cytokine antagonists that exhibit greater effectiveness in blocking the action of the respective cytokine. Polynucleotides that encode improved cytokine antagonists can be obtained by using polynucleotide (e.g. gene, promoter, enhancer, intron, & the like) reassembly (optionally in combination with other directed evolution methods described herein) to generate a recombinant library of polynucleotides which are then screened to identify those that encode an improved antagonist. As substrates for the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly, one can use, for example, polynucleotides that encode receptors for the respective cytokine. At least two forms of the substrate will be present in the reassembly (&/or one or more additional directed evolution methods described herein) reaction, with each form differing from the other in at least one nucleotide position. In one aspect, the different forms of the polynucleotide are homologous cytokine receptor genes from different organisms. The resulting library of experimentally generated polynucleotides is then screened to identify those that encode cytokine antagonists with the desired affinity and biological activity.

As one example of the type of effect that one can achieve by including a cytokine antagonist in a genetic vaccine cocktail, as well as how the effect can be improved using the stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic

polynucleotide reassembly methods of the invention, IL-10 is discussed. The same rationale can be applied to obtaining and using antagonists of other cytokines. Interleukin-10 (IL-10) is perhaps the most potent anti-inflammatory cytokine known to date. IL-10 inhibits a number of pathways that potentiate inflammatory responses. The biological activities of IL-10 include inhibition of MHC class II expression on monocytes, inhibition of production of IL-1, IL-6, IL-12, TNF-α. by monocytes/macrophages, and inhibition of proliferation and IL- 2 production by T lymphocytes. The significance of IL-10 as a regulatory molecule of immune and inflammatory responses was clearly demonstrated in IL-10 deficient mice.

These mice are growth-retarded, anemic and spontaneously develop an inflammatory bowel disease (Kuhn et al. (1993) Cell 75: 263). In addition, both innate and acquired immunity to *Listeria monocytogenes* were shown to be elevated in IL-10 deficient mice (Dai *et al.* (1997) J Immunol. 158: 2259). It has also been suggested that genetic differences in the levels of IL- 10 production may affect the risk of patients to die from complications meningococcal. infection. Families with high IL-10 production had 20-fold increased risk of fatal outcome of meningococcal. disease (Westendorp et al. (1997) Lancet 349: 170).

IL- 10 has been shown to activate normal and malignant B cells *in vitro*, but it does not appear to be a major growth promoting cytokine for normal B cells *in vivo*, because IL-10 deficient mice have normal levels of B lymphocytes and Ig in their circulation. In fact, there is evidence that IL-10 can indirectly downregulate B cell function through inhibition of the accessory cell function of monocytes. However, IL-10 appears to play a role in the growth and expansion of malignant B cells. Anti-IL-10 monoclonal antibodies and IL- 10 antisense oligonucleotides have been shown to inhibit transformation of B cells by EBV *in vitro*. In addition, B cell lymphomas are associated with EBV and most EBV^+ lymphomas produce high levels of IL-10, which is derived both from the human gene and the homologue of IL-10 encoded by EBV. AIDS-related B cell lymphomas also secrete high levels of IL-10. Furthermore, patients with detectable serum IL-10 at the time of diagnosis of intermediate/high-grade non-Hodgkin's lymphoma have short survival, further suggesting IDID In a role for IL- 10 in the pathogenesis of B cell malignancies.

Antagonizing IL- 10 *in vivo* can be beneficial in several infectious and malignant diseases, and in vaccination. The effect of blocking of IL-10 is an enhancement of immune responses that is independent of the specificity of the response. This is useful in vaccinations and in the treatment of serious infectious diseases. Moreover, an IL-10 antagonist is useful in

the treatment of B cell malignancies which exhibit overproduction of IL-10 and viral IL-10, and it may also be useful in boosting general anti-tumor immune response in cancer patients. Combining an IL-10 antagonist with gene therapy vectors may be useful in gene therapy of tumor cells in order to obtain maximal immune response against the tumor cells. If the reassembly (optionally in combination with other directed evolution methods described herein) of IL-10 results in IL-10 with improved specific activity, this IL-10 molecule would have potential in the treatment of autoimmune diseases and inflammatory bowel diseases. IL-10 with improved specific activity may also be useful as a component of gene therapy vectors in reducing the immune response against vectors which are recognized by memory cells and it may also reduce the immunogenicity of these vectors.

An antagonist of IL-10 has been made by generating a soluble form of IL-10 receptor (sIL-10R; Tan et al. (1995) J Biol. Chem. 270: 12906). However, sIL-10R binds IL-10 with Kd of 560 pM, whereas the wild-type, surface-bound receptor has affinity of 35- 200 pM. Consequently, 150-fold molar excess of sIL-10R is required for half-maximal inhibition of biological function of IL-10. Moreover, affinity of viral IL-10 (IL-10 homologue encoded by Epstein-Barr virus) to sIL-10R is more than 1000 fold less than that of hIL-10, and in some situations, such as when treating EBV-associated B cell malignancies, it may be beneficial if one can also block the function of viral IL-10. Taken together, this soluble form of IL-10R is unlikely to be effective in antagonizing IL-10 *in vivo*.

To obtain an IL-10 antagonist that has sufficient affinity and antagonistic activity to function *in vivo*, stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly can be performed using polynucleotides that encode IL-10 receptor. IL-10 receptor with higher than normal affinity will function as an IL- 10 antagonist, because it strongly reduces the amount of IL-10 available for binding to functional, wild-type IL-10R. In one aspect, IL-10R is experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) using homologous cDNAs encoding IL-10R derived from human and other mammalian species.

An alignment of human and mouse IL-10 receptor sequences is shown, described &/or referenced herein (including incorporated by reference) to illustrate the feasibility of family stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly when evolving IL-10 receptors with improved affinity. A phage library of IL-10 receptor recombinants can be screened for improved binding of

experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) IL-10R to human or viral IL-10. Wild- type IL-10 and/or viral IL- 0 are added at increasing concentrations to demand for higher affinity. Phage bound to IL-10 can be recovered using anti-IL-10 monoclonal antibodies. If desired, the shuffling can be repeated one or more times, after which the evolved soluble IL-10R is analyzed in functional assays for its capacity to neutralize the biological activities of IL- 10/viral IL- 10. More specifically, evolved soluble IL-10R is studied for its capacity to block the inhibitory effects of IL-10 on cytokine synthesis and MHC class II expression by monocytes, proliferation by T cells, and for its capacity to inhibit the enhancing effects of IL-10 on proliferation of B cells activated by anti-CD40 monoclonal antibodies.

An IL-10 antagonist can also be generated by evolving IL-10 to obtain variants that bind to IL-10R with higher than wild-type affinity, but without receptor activation. The advantage of this approach is that one can evolve an IL-10 molecule with improved specific activity using the same methods. In one aspect, IL-10 is experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) using homologous cDNAs encoding IL-10 derived from human and other mammalian species. In addition, a gene encoding viral IL-10 can be included in the reassembly (optionally in combination with other directed evolution methods described herein). A library of IL-10 recombinants is screened for improved binding to human IL-10 receptor. Library members bound to IL-10R can be recovered by anti-IL-10R monoclonal antibodies. This screening protocol is likely to result in IL-10 molecules with both antagonistic and agonistic activities. Because initial screen demands for higher affinity, a proportion of the agonists are likely to have improved specific activity when compared to wild-type human IL-10. The functional properties of the mutant IL-10 molecules are determined in biological assays similar to those described above for ultrahigh-affinity IL- 10 receptors (cytokine synthesis and MHC class II expression by monocytes, proliferation of B and T cells). An antagonistic IL-4 mutant has been previously generated illustrating the general feasibility of the approach (Kruse et al. (1992) EMBO J. 11: 3237-3244). One amino acid mutation in IL-4 resulted in a molecule that efficiently binds to IL-4R a-chain but has minimal IL-4-like agonistic activity.

Another example of an IL-10 antagonist is IL-20/mda-7, which is a 206 amino acid secreted protein. This protein was originally characterized as mda-7, which is a melanoma cell-derived negative regulator of tumor cell growth (Jiang et al. (1995) Oncogene 11: 2477;

(1996) Proc. Nat'l. Acad. Sci. USA 93: 9160). IL-20/mda-7 is structurally related to IL-10, and it antagonizes several functions of IL-10 (Abstract of the 13th European Immunology Meeting, Amsterdam, 22-25 June 1997). In contrast to IL-10, IL-20/mda-7 enhances expression of CD80 (B7-1) and CD86 (B7-2) on human monocytes and it upregulates production of TNF-α and IL-6. IL-20/mda-7 also enhances production of IFN-γ by PHA-activated PBMC. The invention provides methods of improving genetic vaccines by incorporation of IL-20/mda-7 genes into the genetic vaccine vectors. The methods of the invention can be used to obtain IL-20/mda-7 variants that exhibit improved ability to antagonize IL-10 activity.

When a cytokine antagonist is used as a component of DNA vaccine or gene therapy vectors, maximal local effect is desirable. Therefore, in addition to a soluble form of a cytokine antagonist, a transmembrane form of the antagonist can be generated. The soluble form can be given in purified polypeptide form to patients by, for example, intravenous injection. Alternatively, a polynucleotide encoding the cytokine antagonist can be used as a component as a component of a genetic vaccine or a gene therapy vector. In this case, either or both of the soluble and transmembrane forms can be used. Where both soluble and transmembrane forms of the antagonist are encoded by the same vector, the target cells express both forms, resulting in maximal inhibition of cytokine function on the target cell surface and in their immediate vicinity.

The peptides or polypeptides obtained using these methods can substitute for the natural ligands of the receptors, such as cytokines or other costimulatory molecules in their ability to exert an effect on the immune system via the receptor. A potential disadvantage of administering cytokines or other costimulatory molecules themselves is that an autoimmune reaction could be induced against the natural molecule, either due to breaking tolerance (if using a natural cytokine or other molecule) or by inducing cross-reactive immunity (humoral or cellular) when using related but distinct molecules. Through using the methods of the invention, one can obtain agonists or antagonists that avoid these potential drawbacks. For example, one can use relatively small peptides as agonists that can mimic the activity of the natural immunomodulator, or antagonize the activity, without inducing cross-reactive immunity to the natural molecule. In one aspect, the optimized agonist or antagonist obtained using the methods of the invention is about 50 amino acids or length or less, or about 30 amino acids or less, or about 20 amino acids in length, or less. The agonist or

antagonist peptide can beat least about 4 amino acids in length, or at least about 8 amino acids in length. Polynucleotides that flank the coding sequence of the mimetic peptide can also be optimized using the methods of the invention in order to optimize the expression, conformation, or activity of the mimetic peptide.

The optimized agonist or antagonist peptides or polypeptides are obtained by generating a library of experimentally generated polynucleotides and screening the library to identify those that encode a peptide or polypeptide that exhibits an enhanced ability to modulate an immune response. The library can be produced using methods such as stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly or other methods described herein or otherwise known to those of skill in the art. Screening is conveniently conducted by expressing the peptides encoded by the library members on the surface of a population of replicable genetic packages and identifying those members that bind to a target of interest, e.g., a receptor.

The optimized experimentally generated polynucleotides that are obtained using the methods of the invention can be used in several ways. For example, the polynucleotide can be placed in a genetic vaccine vector, under the control of appropriate expression control sequences, so that the mimetic peptide is expressed upon introduction of the vector into a mammal. If desired, the polynucleotide can be placed in the vector embedded in the coding sequence of the surface protein (e.g., geneIII or geneVIII) in order to preserve, the conformation of the mimetic. Alternatively, the mimetic-encoding polynucleotide can be inserted directly into the antigen-encoding sequence of the genetic vaccine to form a coding sequence for a "mimotope-on-antigen" structure. The polynucleotide that encodes the mimotope-on-antigen structure can be used within a genetic vaccine, or can be used to express a protein that is itself administered as a vaccine. As one example of this type of application, a coding sequence of a mimetic peptide is introduced into a polynucleotide that encodes the "M-loop" of the hepatitis B surface antigen (HBsAg) protein. The M-loop is a six amino acid peptide sequence bounded by cysteine residues, which is found at amino acids 139-147 (numbering within the S protein sequence). The M-loop in the natural HBsAg protein is recognized by the monoclonal antibody RFHB7 (Chen et al., Proc. Nat'l. Acad. Sci. USA, 93: 1997-2001 (1996)). According to Chen et al., the M-loop forms an epitope of the HBsAg that is non-overlapping and separate from at least four other HBsAg epitopes.

Because of the probable Cys-Cys disulfide bond in this hydrophilic part of the protein, amino acids 139-147 are likely in a cyclic conformation. This structure is therefore similar to that found in the regions of the filamentous phage proteins pIII and pVIII where mimotope sequences are placed. Therefore, one can insert a mimotope obtained using the methods of the invention into this region of the HBsAg amino acid sequence.

The chemokine receptor CCR6 is an example of a suitable target for a peptide mimetic obtained using the methods. The CCR6 receptor is a 7- transmembrane domain protein (Dieu et al., Biochem. Biophys. Res. Comm. 236: 212-217 (1997) and J. Biol. Chem. 272: 14893-14898 (1997)) that is involved in the chemoattraction of immature dendritic cells, which are found in the blood and migrate to sites of antigen uptake (Dieu et al., J Exp. Med. 188: 373-386 (1998)). CCR6 binds the chemokine MIP-3α, so a mimetic peptide that is capable of activating CCR6 can provide a further chemoattractant function to a given antigen and thus promote uptake by dendritic cells after immunization with the antigen antigen-mimetic fusion or a DNA vector that expresses the antigen.

Another application of this method of the invention is to obtain molecules that can act as an agonist for the macrophage scavenger receptor (MSR; see, Wloch et al., Hum. Gene Ther. 9: 1439-1447 (1998)). The MSR is involved in mediating the effects of various immunomodulators. Among these are bacterial DNA, including the plasmids used in DNA vaccination, and oligonucleotides, which are often potent immunostimulators.

Oligonucleotides of certain chemical structure (e.g., phosphothio- oligonucleotides) are particularly potent, while bacterial or plasmid DNA must be used in relatively large quantities to produce an effect. Also mediated by the MSR is the ability of oligonucleotides that contain dG residues to stimulate B cells and enhance the activity of immunostimulatory CpG motifs, and of lipopolysaccharides to activate macrophages. Some of these activities are toxic. Each of these immunomodulators, along with a variety of polyanionic ligands, binds to the MSR. The methods of the invention can be used to obtain mimetics of one or more of these immunomodulators that bind to the MSR with high affinity but are devoid of toxic properties. Such mimetic peptides are useful as immunostimulators or adjuvants.

The MSR is a trimeric integral membrane glycoprotein. The three extracellular C-terminal cysteine-rich regions are connected to the transmembrane domain by a fibrous region that is composed of an (x-helical coil and a collagen-like triple helix (see, Kodama et al., Nature 343: 531-535 (1990)). Therefore, screening of the library of experimentally

generated polynucleotides can be accomplished by expressing the extracellular receptor structure and artificially attaching it to plastic surfaces. The libraries can be expressed, e.g., by phage display, and screened to identify those that bind to the receptors with high affinity.

The optimized experimentally generated polynucleotides identified by this method can be incorporated into antigen-encoding sequences to evaluate their modulatory effect on the immune response.

Costimulatory Molecules Capable Of Inhibiting Or Enhancing Activation, Differentiation, Or Anergy Of Antigen-Specific T Cells

Also provided are methods of obtaining optimized experimentally generated polynucleotides that, when expressed, are capable of inhibiting or enhancing the activation, differentiation, or anergy of antigen-specific T cells. T cell activation is initiated when T cells recognize their specific antigenic peptides in the context of MHC molecules on the plasma membrane of antigen presenting cells (APC), such as monocytes, dendritic cells (DC), Langerhans cells or B cells. Activation of $CD4^+$ T cells requires recognition by the T cell receptor (TCR) of an antigenic peptide in the context of MHC class II molecules, whereas $CD8^+$ T cells recognize peptides in the context of MHC class I molecules.

Importantly, however, recognition of the antigenic peptides is not sufficient for induction of T cell proliferation and cytokine synthesis. An additional costimulatory signal, "the second signal", is required. The costimulatory signal is mediated via CD28, which binds to its ligands B7-1 (CD80) or B7-2 (CD86), typically expressed on the antigen presenting cells. In the absence of the costimulatory signal, no T cell activation occurs, or T cells are rendered anergic. In addition to CD28, CTLA-4 (CD152) also functions as a ligand for B7-1 and B7- 2. However, in contrast to CD28, CTLA-4 mediates a negative regulatory signal to T cells and/or to induce anergy and tolerance (Walunas et al. (1994) Immunity 1: 405; Karandikar et al. (1996) J Exp. Med. 184: 783).

B7-1 and B7-2 have been shown to be able to regulate several immunological responses, and they have been implicated to be of importance in the immune regulation in vaccinations, allergy, autoimmunity and cancer. Gene therapy and genetic vaccine vectors expressing B7-1 and/or B7-2 have also been shown to have therapeutic potential in the treatment of the above mentioned diseases and in improving the efficacy of genetic vaccines.

Figure 39 illustrates interaction of APC and $CD4^+$ T cells, but the same principle is true with $CD8^+$ T cells, with the exception that the T cells recognize the antigenic peptides in

the context of MHC class I molecules. Both B7-1 and B7-2 bind to CD28 and CTLA-4, even though the sequence similarities between these four molecules are very limited (20-30%). It is desirable to obtain mutations in B7-1 and B7-2 that only influence binding to one ligand but not to the other, or improve activity through one ligand while decreasing the activity through the other. Moreover, because the affinities of B7 molecules to their ligands appear to be relatively low, it would also be desirable to find mutations that improve/alter the activities of the molecules. However, rational design does not enable predictions of useful mutations because of the complexity of the molecules.

The invention provides methods of overcoming these difficulties, enabling one to generate and identify functionally different B7 molecules with altered relative capacities to induce T cell activation, differentiation, cytokine production, anergy and/or tolerance. Through use of the methods of the invention, one can find mutations in B7-1 and B7-2 that only influence binding to one ligand but not to the other, or that improve activity through one ligand while decreasing the activity through the other by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is likely to be the most powerful method in discovering new B7 variants with altered relative binding capacities to CD28 and CTLA-4. B7 variants which act through CD28 with improved activity (and with decreased activity through CTLA-4) are expected to have improved capacity to induce activation of T cells. In contrast, B7 variants which bind and act through CTLA-4 with improved activity (and with decreased activity through CD28) are expected to be potent negative regulators of T cell functions and to induce tolerance and anergy.

Stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly or other reassembly (&/or one or more additional directed evolution methods described herein) method is used to generate B7 (e.g., B7-1/CD80 and B7-2/CD86) variants which have altered relative capacity to act through CD28 and CTLA-4 when compared to wild-type B7 molecules. In one aspect, the different forms of substrate used in the reassembly (&/or one or more additional directed evolution methods described herein) reaction are B7 cDNAs from various species. Such cDNAs can be obtained by methods known to those of skill in the art, including RT-PCR. Typically, genes encoding these variant B7 molecules are incorporated into genetic vaccine vectors encoding an antigen, so that one the vectors can be used to modify antigen-specific T cell responses. Vectors that harbor B7 genes that efficiently act through CD28 are useful in inducing, for

example, protective immune responses, whereas vectors that harbor genes encoding B7 genes that efficiently act through CTLA-4 are useful in inducing, for example, tolerance and anergy of allergen- or autoantigen- specific T cells. In some situations, such as in tumor cells or cells inducing autoimmune reactions, the antigen may already be present on the surface of the target cell, and the variant B7 molecules may be transfected in the absence of additional exogenous antigen gene. A screening protocol that one can use to identify B7-1 (CD80) and/or B7-2 (CD86) variants that have increased capacity to induce T cell activation or anergy is diagrammed herein, and the application of this strategy is described in more detail herein.

Several approaches for screening of the variants can be taken. For example, one can use a flow cytometry-based selection systems. The library of B7- 1 and B7-2 molecules is transfected into cells that normally do not express these molecules (e.g., COS-7 cells or any cell line from a different species with limited or no cross- reactivity with man regarding B7 ligand binding). An internal marker gene can be incorporated in order to analyze the copy number per cell. Soluble CTLA-4 and CD28 molecules can be generated to for use in the flow cytometry experiments. Typically, these will be fused with the Fc portion of IgG molecule to improve the stability of the molecules and to enable easy staining by labeled anti-IgG mAbs, as described by van der Merwe et al. (J. Exp. Med: 185: 393, 1997). The cells transfected with the library of B7 molecules are then stained with the soluble CTLA-4 and CD28 molecules. Cells demonstrating increased or decreased CTLA- 4/CD28 binding ratio will be sorted. The plasmids are then recovered and the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) B7 variant-encoding sequences identified. These selected B7 variants can then be subjected to new rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection, and/or they can be further analyzed using functional assays as described below.

The B7 variants can also be directly selected based on their functional properties. For *in vivo* studies, the B7 molecules can also be evolved to function on mouse cells. Bacterial colonies with plasmids with mutant B7 molecules are picked and the plasmids are isolated. These plasmids are then transfected into antigen presenting cells, such as dendritic cells, and the capacities of these mutants to activate T cells is analyzed. One of the advantages of this approach is that no assumptions on the binding affinities or specificities to the known ligands

are made, and possibly new activities through yet to be identified ligands can be found. In addition to dendritic cells, other cells that are relatively easy to transfect (e.g., U937 or COS-7) can be used in the screening, provided that the "first T cell signal" is induced by, for example, anti-CD3 monoclonal antibodies. T cell activation can be analyzed by methods known to those of skill in the art, including, for example, measuring proliferation, cytokine production, CTL activity or expression of activation antigens such as IL-2 receptor, CD69 or HLA-DR molecules. Usage of antigen-specific T, cell clones, such as T cells specific for house dust mite antigen Der p I, will allow analysis of antigen-specific T cell activation (Yssel et al. (1992) J Immunol. 148: 738-745). Mutants are identified that can enhance or inhibit T cell proliferation or enhance or inhibit CTL responses. Similarly variants that have altered capacity to induce cytokine production or expression of activation antigens as measured by, for example, cytokine- specific ELISAs or flow cytometry can be identified.

The B7 variants are useful in modulating immune responses in autoimmune diseases, allergy, cancer, infectious disease and vaccination. B7 variants which act through CD28 with improved activity (and with decreased activity through CTLA-4) will have improved capacity to induce activation of T cells. In contrast, B7 variants which bind and act through CTLA-4 with improved activity (and with decreased activity through CD28) will be potent negative regulators of T cell functions and to induce tolerance and anergy. Thus, by incorporating genes encoding these variant B7 molecules into genetic vaccine vectors encoding an antigen, it is possible to modify antigen-specific T cell responses. Vectors that harbor B7 genes that efficiently act through CD28 are useful in inducing, for example, protective immune responses, whereas vectors that harbor genes encoding B7 genes that efficiently act through CTLA-4 are useful in inducing, for example, tolerance and anergy of allergen- or autoantigen-specific T cells. In some situations, such as in tumor cells or cells inducing autoimmune reactions, the antigen may already be present on the surface of the target cell, and the variant B7 molecules may be transfected in the absence of additional exogenous antigen gene.

The methods of the invention are also useful for obtaining B7 variants that have increased effectiveness in directing either T_H1 or T_H2 cell differentiation. Differential roles have been observed for B7-1 and B7-2 molecules in the regulation of T helper (T_H) cell differentiation (Freeman et al. (1995) Immunity 2: 523; Kuchroo et al. (1995) Cell 80: 707). T_H cell differentiation can be measured by analyzing, the cytokine production profiles

induced by each particular variant. High levels of IL-4, IL-5 and/or IL-13 are an indication of efficient T_H2 cell differentiation whereas high levels of IFN-γ or IL-2 production can be used as a marker of T_H1 cell differentiation. B7 variants with altered capacity to induce T_H1 or T_H2 cell differentiation are useful, for example, in the treatment of allergic, malignant, autoimmune and infectious diseases and in vaccination.

Also provided by the invention are methods of obtaining B7 variants that have enhanced capacity to induce IL-10 production by antigen-specific T cells. Elevated production of IL-10 is a characteristic of regulatory T cells, which can suppress proliferation of antigen-specific $CD4^+$ T cells (Groux et al. (1997) Nature 389: 737). stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is performed as described above, after which recombinant nucleic acids encoding B7 variants having enhanced capability of inducing IL-10 can be identified by, for example, ELISA or flow cytometry using intracytoplasmic cytokine staining. The variants that induce high levels of IL-10 production are useful in the treatment of allergic and autoimmune diseases.

Evolution Of Genetic Vaccine Vectors For Increased Vaccination Efficacy And Ease Of Vaccination

This section discusses the application of the invention to some specific goals in genetic vaccination. Many of these goals relate to improvements in vectors used in vaccine delivery. Unless otherwise indicated the methods are applicable to both viral and nonviral vectors.

Topical Application Of Genetic Vaccine Vectors

Low efficiency of topical application; protective immune responses have not been demonstrated

The invention provides methods of improving the ability of genetic vaccine vectors to induce a desired response after topical application of the vector. Adenoviral vectors topically applied to bare skin have been shown to be capable of acting as vaccine antigen delivery vehicles (Tang et al. (1997) Nature 388: 729-730). An adenoviral vector that encoded carcinoembryonic antigen (CA) was shown to induce antibodies specific for CA after application to the skin. However, the efficiency of topical application is generally quite low, and protective immune responses have not been demonstrated after topical application.

Optimizing the topical application efficiency using the methods of the invention

The invention provides methods of obtaining vectors that exhibit improved efficiency when topically administered. Several factors can influence topical application efficiency, each of which can be optimized using the methods of the invention. For example, the invention provides methods of improving vector affinity for skin cells, improved skin cell transfection efficiency, improved persistence of the vector in skin cells (both through improved replication or through avoidance of destruction by immune cells), and improved antigen expression in skin cells, and improved induction of an immune response.

Methods of reassembly (optionally in combination with other directed evolution methods described herein), selection, and screening

These methods involve performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly using as substrates plasmid, naked DNA vectors, or viral vector nucleic acids, including, for example, adenoviral vectors. Libraries of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) nucleic acids are screened to identify those nucleic acids that confer upon a vector an enhanced ability to induce an immune response upon topical administration. Screening can be conducted by, for example, topically applying a library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) vectors to skin, either mouse skin, monkey skin, or human skin that has been transplanted to immunodeficient mice, or to normal human skin *in vivo*. Vectors that persist and/or provide efficient and long-lasting expression of marker gene are recovered from the skin samples. In a preferred embodiment, the desired cells are first selected by cell sorting, magnetic beads, or panning. For example, recovery can be effected through expression of a marker gene (e.g., GFP) and detecting cells that are transfected using fluorescence microscopy or flow cytometry. Cells that express the marker gene can be isolated using flow cytometry based cell sorting. Screening can also involve selection of vectors that induce the highest specific antibody or CTL responses upon administration to a test mammal, or the identification of vectors that provide an enhanced protective immune response to challenge with a corresponding pathogen. Experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides are then recovered, e.g., by polymerase chain reaction, or the entire vectors can be purified from these selected cells. If desired, further optimization of topical application efficiency can be obtained by subjecting the recovered experimentally evolved (e.g. by polynucleotide

reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides to new rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection.

Administration of genetic vaccine vectors optimized for topical application

Genetic vaccine vectors that are optimized for topical application can be applied topically to the skin, or by intramuscular, intravenous, intradermal, oral, anal, or vaginal delivery. The vector can be delivered in any of the suitable forms that are known to those of skill in the art, such as a patch, a cream, as naked DNA, or as a mixture of DNA and one or more transfection-enhancing agents such as liposomes and/or lipids. In one aspect,, the genetic vaccine vector is applied after the skin or other target is rendered more susceptible to uptake of the vector by, for example, mechanical abrasion, removal of hair (e.g., by treatment with a commercially available product such as Nair™, Neet™, and the like). In one embodiment, the skin is pretreated with proteases or lipases to make it more susceptible to DNA delivery. In addition, the DNA can be mixed with the proteases or lipases to enhance gene transfer. Alternatively, a droplet containing the vector and other vaccine components, if any, can simply be administered to the skin.

Enhanced Ability To Escape Host Immune System

Limitations of host immune responses directed against the viral vector sometimes even before target cells are entered

Immunogenicity is a particular concern with viral vectors, since a host immune response can prevent a virus from reaching its intended target particularly in repeated administrations. The efficacy of some viral vectors which are used for genetic vaccination and gene delivery is limited by host immune responses directed against the viral vector. For example, most individuals have pre-existing antibodies against adenovirus. Adenoviral vectors can sometimes induce strong immune responses which can destroy cells harboring adenoviral vectors or clear adenoviral vectors from the host even before target cells are entered. Cellular immune responses can also be induced against nonviral vectors administered in naked form or shielded with a coat such as liposomes.

Methods to create genetic vaccine vectors with improved ability to avoid the humoral and cellular immune systems

The invention provides methods to create genetic vaccine vectors that can escape immune responses that would otherwise be detrimental to obtaining the desired effect. These

methods are useful for prolonging expression and secretion of pathogen antigen or pharmaceutically useful protein by genetic vaccine vectors. Several strategies are provided by which one can improve a genetic vaccine vector's ability to avoid the humoral (Ab) and cellular (CTL) immune systems. These strategies can be used in combination to obtain optimal avoidance such as may be required for highly immunogenic vectors such as adenovirus.

Incorporating into genetic vaccines one or more components that inhibit peptide transport and/or MHC class I expression in order to obtain viral vectors that are capable of escaping a host CTL immune response

In one embodiment, the invention provides methods of obtaining viral vectors that are capable of escaping a host CTL immune response. This method can be used in conjunction with methods for obtaining genetic vaccine vectors that can escape the humoral response; the combination of approaches is often desirable, as different viral serotypes often have CTL epitopes in common, suggesting that virus variants which are not recognized by antibodies still are likely to be recognized by CTLs. This embodiment of the invention involves incorporating into genetic vaccines one or more components that inhibit peptide transport and/or MHC class I expression. An essential element in the activation of cytotoxic T lymphocyte (CTL) responses is an interaction between T cell receptors on CTLs and antigenic peptide-MHC class I molecule complexes on antigen presenting cells. Expression of MHC class I molecules on thymocytes and antigen presenting cells is a requirement for maturation and activation of antigen-specific $CD8^+$ T lymphocytes. Thus, genes that encode inhibitors of MHC class I-mediated antigen presentation can be experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) as described herein and placed into viral vectors to obtain vectors that, when present in target cells, do not induce destruction of the target cells by the cells of the immune system. This can result in prolonged survival of cells harboring genetic vaccine vectors, including those that express a pathogen antigen, as well as vectors that express a pharmaceutically useful protein. In the case of genetic vaccines, reduced expression of MHC class I molecules will allow secretion of the pathogen antigen, which then will be presented by professional antigen presenting cells elsewhere. In the case of vectors encoding pharmaceutical proteins, reduced expression of MHC class I molecules prevents recognition by the immune system prolonging the survival of the cells expressing the gene.

Reassembly (optionally in combination with other directed evolution methods described herein) genes that encode inhibitors of TAP activity to obtain genes that encode optimized TAP inhibitors

Among the proteins involved in MHC class I molecule expression and antigen presentation are those encoded by TAP genes (transporters associated with antigen processing), which are described above. In one embodiment of the invention, genes that encode inhibitors of TAP activity are experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) to obtain genes that encode optimized TAP inhibitors. The substrates for these methods can include, for example, one or more of the viral genes that are known to regulate levels of MHC class I molecule expression. TAP I and TAP2 gene expression is 5-10-fold and 100-fold reduced, respectively, in cells transformed by adenovirus 12, which results in reduced class I expression and thus leads to reduced virus-specific cytotoxic T lymphocyte responses. Similarly, TAP gene expression is downregulated in 49% of HPV-16$^+$ cervical carcinomas (Seliger et al. (1997) Immunol. Today 18: 292). Thus, adenovirus and HPV viral nucleic acids provide examples of suitable substrates for carrying out the methods of the invention. Additional examples of suitable stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly substrates for this embodiment of the invention include the human cytomegalovirus (CMV) encoded genes US2, US3 and US 11, which can downregulate MHC class I expression (Wiertz et al. (1996) Nature 384: 432 and Cell (1996) 84: 769; Ahn et al. (1996) Proc. Nat'l. Acad Sci. USA 93: 10990). Another human CMV gene that encodes an inhibitor of TAP-dependent peptide translocation is US6 (Lehner et al. (1997) Proc. Nat'l. Acad Sci. USA 94: 6904-9). Cells transfected with US6 had reduced expression of MHC class I molecules on their surface and reduced capacity to activate cytotoxic T lymphocytes.

Reassembly (optionally in combination with other directed evolution methods described herein) this 7kb cluster of genes in order to find the most potent sequence for inhibiting the expression of MHC class I molecules, which can also be used for generation of animal models

Thus, in one embodiment, the invention involves stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly of this cluster of genes (approximately 7kb), or fragments thereof, in order to identify the sequences that are most potent in inhibiting the expression of MHC class I molecules. Such optimized

TAP inhibitor polynucleotide sequences are useftil not only for use in constructing vectors that can escape CTL immune responses, but also for generation of animal models for use with human viruses that normally are eliminated in laboratory animals due to their immunogenicity. The desired expression levels and functional properties of TAP inhibitors may vary depending on whether genetic vaccine vector, gene therapy vector or animal model is evolved.

Reassembly (optionally in combination with other directed evolution methods described herein) other genes involved in downregulating expression of MHC class I molecules and/or antigen presentation

Alternative embodiments of the invention involve stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly of other genes that are involved in downregulating expression of MHC class I molecules and/or antigen presentation. Examples of other possible target genes include genes encoding adenoviral E3 protein, herpes simplex ICP47 protein, and tapasin antagonists (Seliger et al. (1997) Immunol. Today 18:292-299; Galoncha et al. (1997) J Exp. Med. 185: 1565-1572; Li et al. (1997) Proc. Nat'l. Acad. Sci. USA 94: 8708-8713; Ortmann et al. (1997) Science 277: 1306-1309.

A gene that encodes an MHC-like molecule that inhibits NK cell function but is unable to present antigens to T lymphocytes

Because reduced expression of MHC class I molecules on cell surfaces may act as a stimulus for NK cells, it may be useful to include in genetic vaccine vectors a gene that encodes an MHC like molecule that inhibits NK cell function but is unable to present antigens to T lymphocytes. An example of such molecule is MHC class I homologue encoded by cytomegalovirus (Farrell et al. (1997) Nature 3 86: 510-514).

Obtaining viral vectors that exhibit an enhanced capability of avoiding attack by CD4+ T lymphocytes

The invention also provides methods of obtaining viral vectors that exhibit an enhanced capability of avoiding attack by $CD4^+$ T lymphocytes. Such vectors are particularly useful in situations where the target cells are capable of expressing MHC class II molecules, such as in the case of vaccinations and gene therapy targeted to the cells of the immune system. Substrates for stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly include genes that encode inhibitors of MHC class

II molecules such as, for example, IL-10 and antagonists of IFN-γ (such as soluble IFN-γ receptor).

Improving sequences that result in inhibition of MHC class I expression, MHC class II expression, and additional sequences that encode homologs of MHC class I molecules

Vectors that have the greatest capability of escaping the host immune system, will typically include DNA sequences that result in inhibition of MHC class I expression and MHC class II expression, and additional sequences that encode homologs of MHC class I molecules. The properties of all these can be further improved by stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly according to the methods of the invention.

Methods for screening the library to identify those polynucleotides that exhibit the desired effect on the host immune response

Once a library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) DNA molecules is obtained, any of several methods are available for screening the library to identify those polynucleotides that, when present in a viral vector (or in an animal model) exhibit the desired effect on the host immune response. For example, to obtain experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides that inhibit MHC class I expression and/or antigen presentation, a library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) genes can be incorporated into genetic vaccine or gene therapy vectors and transfected into human cell lines, such as, for example, HeLa, U937 or Jijoye, in a single tube transfection. Primary human monocytes, or dendritic cells generated by culturing human cord blood cells or monocytes in the presence of IL-4 and GM-CSF, are also suitable. Initial screening can be done using FACS-sorting.

Cells expressing the lowest levels of MHC class I molecules are expected to have the lowest capacity to induce CTL responses

Cells expressing the lowest levels of MHC class I molecules are selected, the polynucleotides that encode the MHC inhibitors, or whole plasmids containing the sequences, are recovered. If desired, the selected sequences can be subjected to new rounds of reassembly (optionally in combination with other directed evolution methods described

herein) and selection. Cells expressing the lowest levels of MHC class I molecules are expected to have the lowest capacity to induce CTL responses.

Screening method: injecting library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides that encode inhibitors of MHC class I expression incorporated into HPV vectors

Another screening method involves incorporating libraries of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides that encode inhibitors of MHC class I expression are incorporated into human papillomavirus (HPV) vectors. This library is injected into the skin of mice.

Normally, murine cells expressing HPV are destroyed by the host immune system. Cells expressing potent inhibitors of peptide transportation and/or MHC class expression will be able to escape the immune response

However, cells expressing potent inhibitors of peptide transportation and/or MHC class expression will be able to escape the immune response. The cells that express a marker gene present on the vector, such as GFP, for extended periods of time are selected, the sequences or whole plasmids are recovered, and, if further optimization is desired, the selected sequences are subjected to new rounds of reassembly (optionally in combination with other directed evolution methods described herein) and selection. Long- lasting maintenance of HPV in mice will allow drug screening and vaccine studies, which to date have not been possible due to high immunogenicity of HPV in mice.

Evolved inhibitors will block efficient presentation of immunogenic peptides, and hence, will strongly downregulate activation of antigen-specific CTLs allowing long-lasting transgene expression *in vivo*

In another embodiment, the libraries of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) polynucleotides encoding inhibitors of MHC class I expression are incorporated into human adenovirus vectors. This library is transfected into human cell lines, such as HeLa cells, and cells expressing the lowest levels of MHC class I molecules are selected as described above. The sequences that provide the lowest levels of MHC class I expression are further tested by analyzing the capacity of antigen-presenting cells transfected with adenovirus harboring evolved inhibitors of MHC class I expression to activate specific T cell lines or clones. These inhibitors will block efficient presentation of immunogenic peptides, and hence, will strongly

downregulate activation of antigen-specific CTLs allowing long-lasting transgene expression *in vivo*.

Methods to screen for inhibitors

Methods to screen for improved inhibitors of MHC class II expression include detection of MHC class II molecules on the surface of the target cells by fluorescent labeled specific monoclonal antibodies, fluorescence microscopy, and flow cytometry. In addition, the inhibitors can be analyzed in functional assays by studying the capacity of the inhibitors to block activation of MHC class II restricted antigen- specific $CD4^+$ T lymphocytes. For example, one can determine the capacity of the inhibitor to inhibit induction of $CD4^+$ T cell proliferation induced by autologous antigen presenting cells, such as monocytes, dendritic cells, B cells or EBV-transformed B cell lines, that harbor genes encoding the MHC class II inhibitor or have been treated with supernatant containing the inhibitor.

Enhanced Antiviral Activity

Obtaining a recombinant viral vector which has an enhanced ability to induce an antiviral response in a cell

The invention also provides methods of obtaining a recombinant viral vector which has an enhanced ability to induce an antiviral response in a cell. These methods can include the steps of:

(1) reassembling (&/or subjecting to one or more directed evolution methods described herein) at least first and second forms of a nucleic acid which comprise a viral vector, wherein the first and second forms differ from each other in two or more nucleotides, to produce a library of recombinant viral vectors;

(2) transfecting the library of recombinant viral vectors into a population of mammalian cells;

(3) staining the cells for the presence of Mx protein; and

(4) isolating recombinant viral vectors from cells which stain positive for Mx protein, wherein recombinant viral vectors from positive staining cells exhibit enhanced ability to induce an antiviral response.

Stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly is used to produce a library of recombinant viral vectors. The library is transfected into a population of mammalian cells, which are then tested for ability to induce an antiviral response. One suitable test involves staining the cells for the presence

of Mx protein, which is produced by cells that are exhibiting an antiviral response (see, e.g. , Hallimen et al. (1997) Pediatric Research 41: 647-650; Melen et al. (1994) J Biol. Chem. 269: 2009-2015).

Recombinant viral vectors can be isolated from cells which stain positive for Mx protein. These recombinant viral vectors from positive staining cells are enriched for those that exhibit enhanced ability to induce an antiviral response. Viral vectors for which this method is useful include, for example, influenza virus.

Evolution Of Vectors Having Increased Copy Number In Production Cells

Desirability of method to increase the plasmid copy number after all elements have been cloned in the vector, especially when the plasmid is to be manufactured on a large scale

The invention provides methods for obtaining vector components that, when present in a genetic vaccine vector (such as a plasmid) the ability to replicate to a high copy number in a cell used to produce the vector. Plasmids can incorporate various heterologous DNA sequences, however the size or the nature of the cloned sequences in a given plasmid vector may render that vector less able to grow to high copy number in the bacteria in which it is propagated. It is therefore desirable to have a method to increase the plasmid copy number after all elements have been cloned into the vector. This is especially important when the plasmid is to be manufactured on a large scale as will be the case for genetic vaccines.

Incorporating into the plasmid one or more polynucleotide sequences that bind proteins which would otherwise be toxic to the bacterium

The methods of the invention involve incorporating into the plasmid one or more polynucleotide sequences that bind proteins which would otherwise be toxic to the bacterium. One suitable toxic moiety and binding site combination is the transcription factor GATA-1 and its recognition site. It has been shown that expression of a DNA-binding fragment of GATA-1 is toxic to bacteria; this toxicity apparently results from inhibition of bacterial DNA replication. Trudel et al. ((1996) Biotechniques 20: 684- 693) have described a plasmid (pGATA) that expresses the Z2B2 region of GATA-1 as a GST fusion protein. The expression of the fusion protein in this plasmid is under the control of the IPTG-inducible lac promoter. The GST-GATA-1 fragment also binds strongly to a sequence from the mouse β-globin gene promoter as well as to the C-oligonucleotide from the β-globin gene 3' enhancer; either or both of these are suitable for use as binding sites in the methods of the invention.

Including only a single form of the selectable marker in the shiffling reaction to achieve significant diversity in the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) library to recover a plasmid which is improved in its growth properties while fully retaining the appropriate selection function of the plasmid

The plasmids can also include a selectable marker such as, for example, kanamycin resistance (aminoglycoside 3'-phosphotransferase (EC 2.7.1.95)) and the like. The plasmid backbone polynucleotide sequence is subjected to stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly as described herein to generate a library of plasmids which have different backbone sequences and possibly different supercoil densities. In order to introduce sufficient sequence diversity to search for improved function, family stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly can be performed. This can be accomplished in the context of the present invention by including in the reassembly (optionally in combination with other directed evolution methods described herein) reaction(s) only a single form of the selectable marker. In this way, significant diversity can be achieved in the experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) library to recover a plasmid which is improved in its growth properties while fully retaining the appropriate selection function of the plasmid.

Selecting for high copy number plasmids

The selection for high copy number plasmids is performed by introducing the library of experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) recombinant plasmids into the desired host cell. The host cells can also express the toxic moiety, and, in one aspect, under the control of a promoter which is inducible. For example, the pGATA plasmid is suitable for use in E. coli host cells. The experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) plasmids are introduced into the cells under non-inducing conditions. Transformed cells are then placed under conditions which induce expression of the toxic moiety. For example, E. coli cells that contain pGATA can be placed on media containing increasing concentrations of IPTG. Those target plasmids which grow to high copy number in the bacteria will express correspondingly higher numbers of the binding sequences for GATA-1. The target plasmids will bind the GST-GATA-1 fusion protein and thus neutralize the toxic effects on the bacteria.

Plasmids with the highest copy number are detected as those which confer the best growth to bacteria on the inducer-containing growth media. Such plasmids can be recovered and transformed into bacteria which lack the gene that encodes the toxic moiety; these plasmids should retain their high copy number characteristics. Further rounds of reassembly (optionally in combination with other directed evolution methods described herein) can be used to isolate high copy number plasmids by the above selection procedure. Alternatively, manual screening can be done in the bacterial host of choice, lacking the toxic moiety-encoding plasmid, to avoid any effects due to the presence of this extraneous plasmid.

Optimization Of Transport And Presentation Of Antigens

The invention also provides methods of obtaining genetic vaccines and accessory molecules that can improve the transport and presentation of antigenic peptides. A library of experimentally generated polynucleotides is created and screened to identify those that encode molecules that have improved properties compared to the wild-type counterparts. The polynucleotides themselves can be used in genetic vaccines, or the gene products of the polynucleotides can be utilized for therapeutic or prophylactic applications.

Proteasomes

The class I peptides presented on major histocompatibility complex molecules are generated by cellular proteasomes. Interferon-gamma can stimulate antigen presentation, and part of the mechanism of action of interferon may be due to induction of the proteasome beta-subunits LMP2 and LMP7, which replace the homologous beta-subunits Y (delta) and X (epsilon). Such a replacement changes the peptide cleavage specificity of the proteasome and can enhance class I epitope immunogenicity. The Y (delta) and X (epsilon) subunits, as well as other recently discovered proteasome subunits such as the MECL-1 homologue MC14, are characteristic of cells which are not specialized in antigen presentation. Thus, the incorporation into cells by DNA transfer of LMP2, LMP7, MECL-1 and/or other epitope presentation-specific and potentially interferon-inducible subunits can enhance epitope presentation. It is likely that the peptides generated by the proteasome containing the interferon-inducible subunits are transported to the endoplasmic reticulum by the TAP molecules.

The invention provides methods of obtaining proteasomes that exhibit increased or decreased ability to specifically process MHC class I epitopes. According to the methods, stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic

polynucleotide reassembly is used to obtain evolved proteins that can either have new specificities which might enhance the immunogenicity of some proteins and/or enhance the activity of the subunits once they are bound to the proteasome. Because the transition from a non-specific proteasome to a class I epitope-specific proteasome can pass through several states (in which some but not all of the interferon-inducible subunits are associated with the proteasome), many different proteolytic specificities can potentially be achieved. Evolving the specific LMP-like subunits can therefore create new proteasome compositions which have enhanced functionality for the presentation of epitopes.

The methods involve performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly using as substrates two or more forms of polynucleotides which encode proteasome components, where the forms of polynucleotides differ in at least one nucleotide. reassembly (optionally in combination with other directed evolution methods described herein) is performed as described herein, using polynucleotides that encode any one or more of the various proteasome components, including, for example, LMP2, LMP7, MECL-1 and other individual proteasome components that are specifically involved in class I epitope presentation. Examples of suitable substrates are described in, e.g., Stoliwasser et al. (1997) Eur. J Immunol. 27: 1182-1187 and Gaczynska et al. (1996) J Biol. Chem. 271: 17275-17280. In one aspect, polynucleotide reassembly (optionally in combination with other directed evolution methods described herein) is used, in which the different substrates are proteasome component-encoding polynucleotides from different species.

After the reassembly (&/or one or more additional directed evolution methods described herein) reaction is completed, the resulting library of experimentally generated polynucleotides is screened to identify those which encode proteasome components having the desired effect on class I epitope production. For example, the experimentally generated polynucleotides can be introduced into a genetic vaccine vector which also encodes a particular antigen of interest. The library of vectors can then be introduced into mammalian cells which are then screened to identify cells which exhibit increased antigen- specific immunogenicity. Methods of analyzing proteasome activity are described in, for example, Groettrup et al. (1997) Proc. Nat'l. Acad. Sci. USA 94: 8970- 8975 and Groettrup et al. (1997) Eur. J. Immunol. 26: 863-869.

Alternatively, one can use the methods of the invention to evolve proteins which bind strongly to the proteasome but have decreased or no activity, thus antagonizing the proteasome activity and diminishing a cells ability to present class I molecules. Such molecules can be applied to gene therapy protocols in which it is desirable to lower the immunogenicity of exogenous proteins expressed in the cells as a result of the gene therapy, and which would otherwise be processed for class I presentation allowing the cell to be recognized by the immune system. Such high-affinity low-activity LMP- like subunits will demonstrate immuno suppressive effects which are also of use in other therapeutic protocols where cells expressing a non-self protein need to be protected from an immune response.

The specificity of the proteasome and the TAP molecules (discussed below) may have co-evolved naturally. Thus it may be important that the two pathways of the class I processing system be functionally matched. A further aspect of the invention involves performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly simultaneously on the two gene families followed by random combinations of the two in order to discover appropriate matched proteolytic and transport specificities.

Antigen Transport

The invention provides methods of improving transport of antigenic peptides from the cytosolic compartment to the endoplasmic reticulum. and thereby to the cell surface in the context of MHC class I molecules. Enhanced expression of antigenic peptides results in enhanced immune response, particularly in improved activation of $CD8^+$ cytotoxic lymphocytes. This is useful in the development of DNA vaccines and in gene therapy.

In one embodiment, the invention involves evolving TAP-genes (transporters associated with antigen processing) to obtain genes that exhibit improved antigen presentation. TAP genes are members of ATP-binding cassette family of membrane translocators. These proteins transport antigenic peptides to MHC class I molecules and are involved in the expression and stability of MHC class I molecules on the cell surface. Two TAP genes, TAP1 and TAP2, have been cloned to date (Powis et al. (1996) Proc. Nat'l. Acad. Sci. USA 89: 1463-1467; Koopman et al. (1997) Curr. Opin. Immunol. 9: 80-88; Monaco (1995) J Leukocyte Biol. 57: 543-57). TAP1 and TAP2 form a heterodimer and these genes are required for transport of peptides into the endoplasmic reticulum, where they bind to MHC class I molecules. The essential role of TAP gene products in presentation of antigenic

peptides was demonstrated in mice with disrupted TAP genes. TAP1-deficient mice have drastically reduced levels of surface expression of MHC class I, and positive selection of $CD8^+$ T cells in the thymus is strongly reduced. Therefore, the number of $CD8^+$ T lymphocytes in the periphery of TAP-deficient mice is extremely low. Transfection of TAP genes back into these cells restores the level of MHC class I expression.

TAP genes are a good target for polynucleotide (e.g. gene, promoter, enhancer, intron, & the like) reassembly (optionally in combination with other directed evolution methods described herein) because of natural polymorphism and because these genes of several mammalian species have been cloned and sequenced, including human (Beck et al. (1992) J Mol. Biol. 228: 433-441; Genbank Accession No. Y13582; Powis et al., supra.), gorilla TAP1 (Laud et al. (1996) Human Immunol. 50: 91-102), mouse (Reiser et al. (1988) Proc. Nat'l. Acad. Sci. USA 85: 2255- 2259; Marusina et al. (1997) J Immunol, 158: 5251-5256, TAP1: Genbank Accession Nos. U60018, U60019, U60020, U60021, U60022, and L76468-L67470; TAP2: Genbank Accession Nos. U60087, U60088, U6089, U60090, U60091 and U60092), hamster (TAP1, Genbank Accession Nos. AF001154 and AF001157; TAP2, Genbank Accession Nos. AF001 156 and AF001155). Furthermore, it has been shown that point mutations in TAP genes may result in altered peptide specificity and peptide presentation. Also, functional differences in TAP genes derived from different species have been observed. For example, human TAP and rat TAP containing the rTA.P2a allele are rather promiscuous, whereas mouse TAP is restrictive and select against peptides with C-terminal small polar/hydrophobic or positively charged amino acids. The basis for this selectivity is unknown.

The methods of the invention involve performing stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly of TAP1 and TAP2 genes using as substrates at least two forms of TAP1 and/or TAP2 polynucleotide sequences which differ in at least one nucleotide position. In one aspect, TAP sequences derived from several mammalian species are used as the substrates for reassembly (optionally in combination with other directed evolution methods described herein).

Natural polymorphism of the genes can provide additional diversity of substrate. If desired, optimized TAP genes obtained from one round of reassembly (optionally in combination with other directed evolution methods described herein) and screening can be subjected to additional reassembly (optionally in combination with other directed evolution

methods described herein)/screening rounds to obtain further optimized TAP-encoding polynucleotides.

To identify optimized TAP-encoding polynucleotides from a library of recombinant TAP genes, the genes can be expressed on the same plasmid as a target antigen of interest. If this step is limiting the extent of antigen presentation, then enhanced presentation to $CD8^+$ CTL will result. Mutants of TAPs may act selectively to increase expression of a particular antigen peptide fragment for which levels of expression are otherwise limiting, or to cause transport of a peptide that would normally never be transferred into the RER and made available to bind to MHC Class I.

When used in the context of gene therapy vectors in cancer treatment, evolved TAP genes provide a means to enhance expression of MHC class I molecules on tumor cells and obtain efficient presentation of antigenic tumor- specific peptides. Thus, vectors that contain the evolved TAP genes can induce potent immune responses against the malignant cells. Experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) TAP genes can be transfected into malignant cell lines that express low levels of MHC class I molecules usina retroviral vectors or electroporation.

Transfection efficiency can be monitored using marker genes, such as green fluorescent protein, encoded by the same vector as the TAP genes. Cells expressing equal levels of green fluorescent protein but the highest levels of MHC class I molecules, as a marker of efficient TAP genes, are then sorted using flow cytometry, and the evolved TAP genes are then recovered from these cells by, for example, PCR or by recovering the entire vectors.

These sequences can then subjected into new rounds of reassembly (optionally in combination with other directed evolution methods described herein), selection and recovery, if further optimization is desired. Molecular evolution of TAP genes can be combined with simultaneous evolution of the desired antigen. Simultaneous evolution of the desired antigen can further improve the efficacy of presentation of antigenic peptides following DNA vaccination. The antigen can be evolved, using polynucleotide reassembly (optionally in combination with other directed evolution methods described herein), to contain structures that allow optimal presentation of desired antigenic peptides when optimal TAP genes are expressed. TAP genes that are optimal for presentation of antigenic peptides of one given antigen may be different from TAP genes that are optimal for presentation of antigenic

peptide of another antigen. Polynucleotide (e.g. gene, promoter, enhancer, intron, & the like) reassembly (optionally in combination with other directed evolution methods described herein) technique is ideal, and perhaps the only, method to solve this type of problems. Efficient presentation of desired antigenic peptides can be analyzed using specific cytotoxic T lymphocytes, for example, by measuring the cytokine production or CTL activity of the T lymphocytes using methods known to those of skill in the art.

Cytotoxic T-Cell Inducing Sequences And Immunogenic Agonist Sequences

Certain proteins are better able than others to carry MHC class I epitopes because they are more readily used by the cellular machinery involved in the necessary processing for class I epitope presentation. The invention provides methods of identifying expressed polypeptides that are particularly efficient in traversing the various biosynthetic and degradative steps leading to class I epitope presentation and the use of these polypeptides to enhance presentation of CTL epitopes from other proteins.

In one embodiment, the invention provides Cytotoxic T-cell Inducing Sequences (CTIS), which can be used to carry heterologous class I epitopes for the purpose of vaccinating against the pathogen from which the heterologous epitopes are derived. One example of a CTIS is obtained from the hepatitis B surface antigen (HBsAg), which has been shown to be an effective carrier for its own CTL epitopes when delivered as a protein under certain conditions. DNA immunization with plasmids expressing the HBsAg also induces high levels of CTL activity. The invention provides a shorter, truncated fragment of the HBsAg polypeptide which functions very efficiently in inducing CTL activity, and attains CTL induction levels that are higher than with the HBsAg protein or with the plasmids encoding the full-length HBsAg polypeptide. Synthesis of a CTIS derived from HBsAg is described in Example 3; and a diagram of a CTIS is shown, described &/or referenced herein (including incorporated by reference).

The ER localization of the truncated polypeptide may be important in achieving suitable proteolytic liberation of the peptide(s) containing the CTL epitopes (see Cresswell &#0000; Craiu et al. (1997) Proc. Nat'l. Acad. Sei. USA 94: 10850-10855). The preS2 region and the transmembrane region provide T-helper epitopes which may be important for the induction of a strong cytotoxic immune response. Because the truncated CTIS polypeptide has a simple structure, it is possible to attach one or more heterologous class I epitope sequences to the C-terminal end of the polypeptide without having to maintain any specific

protein conformation. Such sequences are then available to the class I epitope processing mechanisms. The size of the polypeptide is not subject to the normal constraints of the native HBsAg structure. Therefore the length of the heterologous sequence and thus the number of included CTL epitopes is flexible. This is shown schematically herein. The ability to include a long sequence containing either multiple and distinct class I sequences, or alternatively different variations of a single CTL sequence, allows stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methodology to be applied.

The invention also provides methods of obtaining Immunogenic Agonist Sequences (IAS) which induce CTLs capable of specific lysis of cells expressing the natural epitope sequence. In some cases, the reactivity is greater than if the CTL response is induced by the natural epitope. Such IAS-induced CTL may be drawn from a T-cell repertoire different from that induced by the natural sequence. In this way, poor responsiveness to a given epitope can be overcome by recruiting T cells from a larger pool. In order to discover such IAS, the amino acid at each position of a CTL-inducing peptide (excluding perhaps the positions of the so-called anchor residues) can be varied over the range of the 19 amino acids not normally present at the position. stochastic (e.g. polynucleotide shuffling & interrupted synthesis) and non-stochastic polynucleotide reassembly methodology can be used to scan a large range of sequence possibilities.

A synthetic gene segment containing multiple copies of the original epitope sequence can be prepared such that each copy possesses a small number of nucleotide changes. The gene segment can be experimentally evolved (e.g. by polynucleotide reassembly &/or polynucleotide site-saturation mutagenesis) to create a diverse range of CTL epitope sequences, some of which should function as IAS. This process is illustrated herein.

In practice, oligonucleotides are typically constructed in accordance with the above design and polymerized enzymatically to form the synthetic gene segment of the concatenated epitopes. Restriction sites can be incorporated into a fraction of the oligonucleotides to allow for cleavage and selection of given size ranges of the concatenated epitopes, most of which will have different sequences and thus will be potential IAS. The epitope-containing gene segment can be joined by appropriate cloning methods to a CTIS, such as that of HBsAg. The resulting plasmid constructions can be used for DNA-based C immunization and CTL induction.

Genetic Vaccine Pharmaceutical Compositions And Methods Of Administration

Using genetic vaccines in prophylaxis and therapy of infectious diseases, autoimmune diseases, other inflammatory conditions, allergies, asthma, and cancer and the prevention of metastasis

The vector components and multicomponent genetic vaccines of the invention are useful for treating and/or preventing various diseases and other conditions. For example, genetic vaccines that employ the reagents obtained according to the methods of the invention are useful in both prophylaxis and therapy of infectious diseases, including those caused by any bacterial, fungal, viral, or other pathogens of mammals. The reagents obtained using the invention can also be used for treatment of autoimmune diseases including, for example, rheumatoid arthritis, SLE, diabetes mellitus, myasthenia gravis, reactive arthritis, ankylosing spondylitis, and multiple sclerosis. These and other inflammatory conditions, including IBD, psoriasis, pancreatitis, and various immunodeficiencies, can be treated using genetic vaccines that include vectors and other components obtained using the methods of the invention. Genetic vaccine vectors and other reagents obtained using the methods of the invention can be used to treat allergies and asthma. Moreover, the use of genetic vaccines have great promise for the treatment of cancer and prevention of metastasis. By inducing an immune response against cancerous cells, the body's immune system can be enlisted to reduce or eliminate cancer.

Use of Recombinant Multivalent Antigens

The multivalent antigens of the invention are useful for treating and/or preventing the various diseases and conditions with which the respective antigens are associated. For example, the multivalent antigens can be expressed in a suitable host cell and are administered in polypeptide form. Suitable formulations and dosage regimes for vaccine delivery are well known to those of skill in the art. The improved immunomodulatory polynucleotides and polypeptides of the invention are useful for treating and/or preventing the various diseases and conditions with which the respective antigens are associated.

An antigen for a particular condition can be optimized using reassembly (&/or one or more additional directed evolution methods described herein) and selection methods analogous to those described herein.

In one aspect, the reagents obtained using the invention (e.g. optimized experimentally generated polynucleotides that encode improved allergens), are used in